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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University College, Gower Street, London, W.C. 1, on 19th March, 1955, at 10.45 a.m. when the following papers were read:*

COMMUNICATIONS

Ruminal Fermentation of Pentosan. By B. H. Howard. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Perlin's (1951*a*) highly purified wheat-flour pentosan was used as substrate. This material contained negligible amounts of N, uronic acids and hexoses, and yielded 105% pentoses on hydrolysis (theoretical 114%). According to Perlin (1951*b*), it consists of a chain of β-1:4-D-xylose units with single L-arabinose units attached along the chain. For the present work this substrate has the advantage of being readily soluble in water.

The total bacteria separated from the rumen contents of a hay and concentrate fed sheep were suspended in $NaHCO_3$ buffer, pH 7·2, and portions mixed with solutions of D-glucose, D-xylose and the pentosan (final concentration 0·1%). 135, 82 and 80 μl./hr. respectively of CO_2 were produced; all fermentations proceeded without lag, yielding volatile fatty acids as the main product. No lactic acid was detected in the pentose or pentosan fermentations (cf. Heald, 1952).

When the pentosan (0·5% solution) was incubated with a suspension of rumen bacteria in presence of toluene or $CHCl_3$, it was rapidly hydrolysed. Stripping off of the arabinose side chains and fragmentation of the xylan chain appeared to proceed concurrently. Arabinose, xylose, xylobiose, xylotriose and xylotetraose have been detected in the hydrolysates by paper chromatography. No low molecular-weight compounds containing both arabinose and xylose were found.

No pentosanase activity was detectable in rumen liquor which had been freed from bacteria even after the fermentation of added pentosan.

When pentosan was added to rumen liquor from which only the larger organisms had been removed by light centrifugation, leaving the small eubacteria, a vigorous fermentation occurred, and 90% of the substrate disappeared during 4 hr. incubation at 38°. Hydrolysis proceeded more rapidly than fermentation of the sugars, so that considerable amounts of arabinose, xylobiose, xylotriose and rather less xylose were found in the early stages. Thus in the overall decomposition of the pentosan the fermentation of the pentoses is the limiting process, a conclusion confirmed by the figures quoted above for rates of CO_2 evolution from xylose and pentosan in Warburg respirometers.

In the early stages of the fermentation there is a substantial accumulation of cellular polysaccharide, which dwindles on further incubation. This material is a glucosan, and at the peak of its concentration it represents about 25% of the pentosan consumed.

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Blood Changes in Rats Treated with Senecio Alkaloids. By Regina Schoental. (*Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow*)

The toxicity of various Senecio alkaloids and the pathological changes in different organs of experimental animals have been studied by several groups of workers (for a review see Sapeika, 1952). However, the blood changes accompanying the various stages of the pathological process have so far not been investigated.

During the experimental study of the induction of a kwashiorkor-like syndrome (Schoental, 1954) liver cirrhosis and primary liver tumours in rats treated with various Senecio alkaloids (Cook, Duffy & Schoental, 1950; Schoental, Head & Peacock, 1954) striking cases of anaemia were encountered, when the red eyes of the albino rats appeared almost colourless.

Consequently data were collected on the various degrees of anaemia and on the variations in total serum protein content, and of the paper electro-

phoretic patterns of protein fractions, at the various stages of the pathological chain of events induced in rats by several Senecio alkaloids.

The causes of the anaemias and hypoproteinaemias (the lowest values encountered were 28% Hb and 3·5 g./100 ml. respectively) will be discussed and correlated with similar findings, reported in the corresponding human disorders (e.g. kwashiorkor) among underdeveloped communities, which are known to use plants containing pyrrolizidine alkaloids in traditional herbal treatments of infants, pregnant women, and at various other occasions.

This work was supported by a grant from the British Empire Cancer Campaign. Protein determinations were by Mrs B. Jack.

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The Optical Configuration of Naturally Occurring Forms of αε-Diaminopimelic Acid. By D. S. Hoare and Elizabeth Work. (*University College Hospital Medical School, London, W.C.* 1)

Samples of αε-diaminopimelic acid (DAP) prepared from two bacterial sources had different optical rotations. DAP from hydrolysed *Corynebacterium diphtheriae* ('diphtheria DAP') had no rotation, whereas that from the culture fluid of a lysine-requiring mutant of *Escherichia coli* ('mutant DAP') had a slight positive rotation and behaved as a mixture of isomers (Work, 1951; Work & Denman, 1953; Wright & Cresson, 1953). Suggestions were made that the *meso* (internally compensated) isomer of DAP was present in both preparations, with some LL-isomer in 'mutant DAP'. Proof has now been obtained for the isomeric forms of DAP in these preparations and in some bacteria. Use was made of the findings that the three isomers obtained by resolution of synthetic DAP (Work, Birnbaum, Winitz & Greenstein, 1955) differ in their behaviour to DAP decarboxylase (Hoare, 1955) and to neurospora L-amino acid oxidase (Work, 1955), and that the LL-isomer separates from the other two isomers on paper chromatograms (Rhuland, 1955; Denman, Hoare & Work, 1955).

'Diphtheria DAP' was identical with the *meso* isomer, both on paper chromatography and on treatment with purified DAP decarboxylase or L-amino acid oxidase. 'Mutant DAP' gave two spots on paper chromatograms and was completely decarboxylated by acetone-dried bacteria which attack LL- and *meso*-DAP. A partially purified decarboxylase preparation, specific for *meso*-DAP, did not completely decarboxylate 'mutant DAP', and the residual DAP behaved as LL-isomer on chromatography and decarboxylation by acetone-dried bacteria. Four recrystallizations from ethanol–water of 'mutant DAP' produced pure *meso*-DAP, as judged by zero rotation, chromatography and decarboxylation by purified enzyme. LL-DAP was obtained by fractionation of the HCl effluent from a Zeocarb-225 column during a normal preparation of 'mutant DAP'; the last quarter of the effluent DAP was crystallized from acetone–HCl and identified as LL-DAP monohydrochloride by analysis and optical rotation, also by chromatographic and enzymic behaviour.

Chromatographic and enzymic examination of DAP in hydrolysates and extracts of bacterial cells showed that while many organisms contained only the *meso*-isomer, others (e.g. *Clostridium welchii* and Propionibacteria) contained only the LL-isomer.

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The Metabolites of 2-Naphthylamine Produced by Rats and Rabbits. By E. Boyland and D. Manson. (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

The examination of urine from rats and rabbits injected with 2-naphthylamine by paper chromatography has revealed the presence of the following compounds: (1) 2-naphthylamine; (2) 2-acetamidonaphthalene; (3) 2-amino-1-naphthylsulphuric acid; (4) 2-amino-1-naphthyl glucosiduronic acid; (5) 2-amino-6-naphthol; (6) 2-acetamido-6-naphthol; (7) 2-amino-6-naphthyl sulphuric acid; (8) 2-

acetamido-6-naphthyl sulphuric acid; (9) 2-naphthylamine-*N*-glucosiduronic acid; (10) 2-naphthyl sulphamic acid; (11) 2-acetamido-6-naphthyl glucosiduronic acid which also occurs in the urine of rabbits injected with 2-amino-6-naphthol and 2-acetamidonaphthalene; (12) a substance which is probably 2-acetamido-5:6-dihydroxy-5:6-dihydronaphthalene which also occurs in the urine of rabbits dosed with 2-acetamidonaphthalene, but not in urine of animals dosed with 2-amino-6-naphthol; (13) a substance which readily yields 2-amino-1-naphthyl sulphuric acid on treatment with acid and might be (*a*) 2-amino-1-naphthyl sulphuric acid -*N*-glucosiduronide, (*b*) *N*-sulphonate (sulphamate) of 2-amino-1-naphthyl sulphate or (*c*) 1-hydroxy-2-naphthylsulphamate.

Substances 5, 7 and 12 have only been detected in rat urine and substance 11 only in rabbit urine, but the other substances are present in the urine of both species.

The sulphamic acid and *N*-glucosiduronic acid derivatives of 2-naphthylamine are new types of metabolites. Sulphamic acid or *N*-glucosiduronic acid derivatives of hydrazobenzene were suggested by Elson & Warren (1944) to account for the properties of a water-soluble substance yielding benzidine on treatment with acid of urine from rats dosed with azobenzene. The formation of *N*-glucosiduronic acids may account for the labile glucosiduronates reported by Smith & Williams (1949*a*, *b*) in a study of the metabolism of aniline and phenetidine. 2-Amino-1-naphthol may also be excreted as derivatives of this type. The *N*-glucosiduronate of 2-naphthylamine is hydrolysed with boiling water and 2-naphthyl sulphamic acid with cold dilute mineral acid. In view of the resistance of 2-amino-1-naphthyl sulphuric acid to hydrolysis by sulphatase (Boyland, Manson, Sims & Williams, 1955) these derivatives of 2-amino-1-naphthol and 2-amino-1-naphthyl glucosiduronic acid may yield the carcinogenic 2-amino-1-naphthol in the bladder, but 2-amino-1-naphthol is not excreted as such.

2-Amino-1-naphthyl phosphate does not appear to be a metabolite of 2-naphthylamine in rats.

2-Acetamido-1-naphthyl glucosiduronic acid has been isolated from urine of rabbits dosed with 2-acetamido-1-naphthol, acid hydrolysis of which yielded 2-amino-1-naphthyl glucosiduronic acid.

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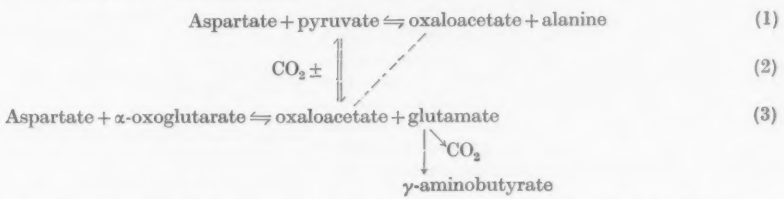
Transaminase Activity in *Clostridium welchii*. By R. Marian Hicks (introduced by Margaret Kerly). (*Department of Biochemistry, University College London*)

Washed suspensions of intact cells of *Clostridium welchii* have been used for the quantitative estimation of L-glutamic acid, by virtue of their specific decarboxylase activity (Gale, 1945; Krebs, 1948). While using this method to determine glutamic acid in a heart perfusate which contained, in addition, aspartic, α-oxoglutaric and oxaloacetic acids, it became apparent that there was a slow steady evolution of gas in addition to the CO_2 produced from the glutamic acid. The source of this extra gas, assumed to be CO_2, was investigated, both by manometric experiments and paper chromatography.

It was found that CO_2 and alanine were produced on incubating aspartic and α-oxoglutaric acids with *Cl. welchii* washed suspensions, but no γ-aminobutyric acid was found as would have been expected if a simple transamination, such as that reported by Lichstein & Cohen (1944), followed by decarboxylation had been taking place. Alanine was also produced by incubating *Cl. welchii* suspensions with aspartic acid and either oxaloacetic or pyruvic acid, but not with aspartic acid alone or together with glucose, succinic, furmaric or malic acids.

It is suggested that the following reactions occur in the incubated mixtures:


Aspartate + pyruvate ⇌ oxaloacetate + alanine (1)
CO_2 ± (2)
Aspartate + α-oxoglutarate ⇌ oxaloacetate + glutamate (3)
CO_2
γ-aminobutyrate

It is suggested that a system analogous to that described by Gale (1947) for *Escherichia coli*, in which glutamic instead of aspartic acid was the transaminating agent.

Since insufficient γ-aminobutyric acid was produced to be detected by the ninhydrin reaction

when aspartic and α-oxoglutaric acids were incubated with *Cl. welchii* suspensions, it may be suggested that the formation of glutamic acid by transamination proceeds less actively than the formation of alanine. If this is true, then only one molecule of oxaloacetic acid need be produced from aspartic acid (reaction 3) and decarboxylated to pyruvic acid (reaction 2) to start the regenerating reaction by which aspartic acid is preferentially converted into alanine (reaction 1).

My thanks are due to Mr R. G. Westall and Mr D. Cusworth of University College Hospital for assistance in the identification of alanine, and to Mrs P. H. Clarke of this department for advice on the preparation and use of the bacteria. This work was done during the tenure of a training grant from the Medical Research Council.

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Urinary Phenolic Acid Excretion in Phenylketonuria. By R. J. Boscott and B. H. Kirman.
(*Department of Anatomy, Medical School Birmingham and Fountain Hospital, Tooting, London*)

Boscott & Bickel (1953) suggested that three metabolic blocks may exist in phenylalanine metabolism in phenylketonuric patients. Compound 30, identified tentatively as *o*-hydroxyphenylacetic acid, has now been isolated in admixture with l-phenyllactic acid as demonstrated by infrared spectroscopy and the production of phenylacetaldehyde upon oxidation with $Ce(SO_4)_2$. Grossly abnormal amounts of *o*-hydroxyphenylacetic acid were found in the urine of all twenty patients studied irrespective of the degree of mental development. The levels of excretion of this compound paralleled the intake of phenylalanine in the diet. Phenyllactic, *p*-hydroxyphenyllactic and lactic acids inhibit the ferric chloride colour reaction for *o*-hydroxyphenylacetic acid. Armstrong has isolated an unusual physical modification of *o*-hydroxyphenylacetic acid from phenylketonuric urines.

In contrast to normals and controls, most phenylketonurics excrete little or no *m*-hydroxyphenylacetic acid. If the enzymic hydroxylation of phenylalanine requires the intermediate formation of a 3:4-glycol, subsequent dehydration (pinacol-pinacolone rearrangement and enolization) would lead to the production of *m*- and *p*-hydroxylated products, whereas a 1:2-glycol would lead similarly only to the production of the *o*-hydroxy compounds. *p*- and *m*-hydroxylation of phenylalanine is apparently much more efficient in normals and controls than in phenylketonurics in whom *o*-hydroxylation preponderates. It is reasonable to suppose that the same prosthetic group is present in the enzyme or enzymes responsible for the three types of hydroxylation. In phenylketonuria the carrier protein of the prosthetic group may possess a grouping which partially prevents *m*- and *p*-hydroxylations by steric hindrance but permits *o*-hydroxylation to take place. These speculations would explain the isotope data of Undenfriend & Bessman (1953).

The alternative explanation (Dalgliesh, 1954) of an incomplete metabolic block by unspecific hydroxylation is an attractive hypothesis, but fails to explain the reversed ratio of *o*- and *m*-hydroxyphenylacetic acids excreted by phenylketonurics compared with normals and controls. Ascorbic acid and iron as *in vivo* hydroxylating agents concerned with the production of *o*-hydroxyphenylacetic acid in humans and guinea pigs is unlikely, in view of the results obtained from ascorbic acid and iron depleted subjects before and after replacement therapy.

These conclusions do not rule out the possibility that combined ascorbic acid may be the prosthetic group of the hydroxylating enzymes having phenylalanine as substrate.

We wish to thank Dr D. H. Whiffen for making several infrared spectral measurements, Miss A. M. Cooke for valuable technical assistance, and Drs H. Bickel, V. Cowie and C. E. Dent for some of the clinical material.

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Structural Requirements for the Inhibition of Amine Oxidases. By E. A. Zeller, J. Barsky, J. R. Fouts and J. C. Lazanas. (*Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois*)

The differentiating characteristics between monoamine oxidase (MO) and diamine oxidase (DO) have been lessened. Certain diamines are substrates of MO (Blaschko & Duthie, 1945) and many monoamines, e.g. tyramine and mescaline, are substrates of DO (Fouts, 1954). Carbonyl reagents such as phenylhydrazine (10^{-3}M) inhibit both amine oxidases (Zeller, Barsky, Fouts, Kirchheimer & Van Orden, 1952). In the cells of some tissues MO and DO are both found in particulate matter. The question arose then as to whether the amine oxidases are distinct entities or are homologous enzymes. The problem has been studied by attempting to analyse the structural requirements for effective inhibitors of MO and DO, employing washed liver mitochondrial preparations of various species, and a partially purified cytoplasmic DO of hog kidney.

Initially, 1 - *iso*nicotinyl - 2 - *iso*propylhydrazine (iproniazid, marsilid) which strongly inhibits MO, and certain forms of DO was used (Zeller *et al.*1952; Zeller & Barsky, 1952). Systematic variation of this structure established the following relationships. The main requirements for inhibitors of MO may be represented by the formula =N—NHR. If R is an aryl or alkyl group, the substitution on the other nitrogen is of less importance. Hydrazine and *NN*-phenylmethylhydrazine are without effect on MO, while methylhydrazine is a potent inhibitor. The carbonyl reagent property is only incidental in MO inhibitors. For blocking of DO the optimal structure is =N—NH_2. Hydrazine inhibits DO at a level of 10^{-6}M; methylhydrazine, phenylhydrazine and *NN*-methylphenylhydrazine are in order of decreasing effectiveness, still excellent inhibitors of DO. Eliminating the carbonyl reagent property of a compound does not completely destroy its DO blocking activity as shown for alkyl and alkylidene derivatives of *iso*nicotinyl-, *iso*nipecotyl- and picolinyl-hydrazines.

These studies have produced a series of new inhibitors of the two amine oxidases, and have clearly shown essential differences in the structural requirements for effective blocking of their respective activities.

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The Estimation of Tryptophan Metabolites in the Urine of Patients with Cancer of the Bladder. By E. Boyland and D. C. Williams. (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

The fact that cancer of the bladder is produced in men by certain external chemical agents suggests that some bladder cancers occurring in man, which have been considered to be spontaneous, are due to unidentified chemical substances derived from the metabolic processes.

Bonser, Clayson, Jull & Pyrah (1952) have shown that a number of *o*-aminophenols are carcinogenic when implanted in the bladder of mice. Among substances present in normal urine are the two *o*-aminophenols, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, which are metabolites of tryptophan. A gradient elution system of column partition chromatography has been developed for the quantitative estimation of these two compounds, their corresponding sulphuric esters, which are naturally occurring conjugates of these substances, and also kynurenine and anthranilic acid which are their precursors. The urine from ten normal subjects and ten patients suffering from cancer of the bladder has been examined.

Both 3-hydroxykynurenine and 3-hydroxyanthranilic acid are present in higher concentrations in the urine from bladder cancer patients than in that from normal subjects. Anthranilic acid and kynurenine are also higher in the bladder cancer group but the sulphuric esters of 3-hydroxyanthranilic acid and 3-hydroxykynurenine are in higher concentrations in normal urine. The difference in the ratio of free phenolic compounds and ethereal sulphuric ester in the two series may be due to the presence of more sulphatase in the urines of the bladder cancer patients (cf. Boyland, Wallace & Williams, 1954).

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The Purification of Hypertensin. By W. S. PEART (introduced by R. R. PORTER). (*Medical Unit, St Mary's Hospital, W. 2, and National Institute for Medical Research, London, N.W. 7*)

Purification of the pressor principle hypertensin or angiotonin (Braun Menendez, Fasciolo, Leloir & Munoz, 1939; Page & Helmer, 1940) has been successful. It was produced by incubation of the enzyme renin from the rabbit kidney with ox serum in the presence of charcoal, from which it was eluted with glacial acetic acid (Peart, 1955).

Pressor activity at all stages was assayed on the anaesthetized rat (Peart, 1955).

Preliminary evidence that hypertensin was likely to be a peptide was obtained by paper electrophoresis at a wide range of pH, which showed that hypertensin was amphoteric, with an isoelectric range from pH 7·0 to 8·5.

(1) The first step in purification was to extract the NaCl saturated solution of hypertensin at pH 1·0 with *n*-butanol (Skeggs, Kahn & Shumway, 1952).

(2) Partition chromatography was next carried out on a Hyflo column holding the lower phase of a mixture of *n*-butanol–acetic acid–water (9:1:10 by vol.), with the top phase mobile.

(3) This activity was next chromatographed on a column of silane-treated Hyflo holding the upper phase of a mixture of *n*-butanol–0·25% trichloroacetic acid (1:4 by vol.), with the lower phase mobile. Two peaks of pressor activity were obtained; the faster running peak had 5% of the total activity.

(4) The main peak was adsorbed on Hyflo and eluted with 0·02 M ammonium formate. Low-pressure distillation of the eluate yielded a white powder, which was 1·5–3·0 times as active wt./wt. as noradrenaline.

Hydrolysis in 6 N-HCl followed by paper chromatography revealed leucine, phenylalanine, valine, tyrosine, proline, aspartic acid, arginine and histidine, with a trace of alanine. Spectrophotometry showed no evidence of tryptophan.

(5) The peptide thus obtained was tested for homogeneity by paper electrophoresis; hydrolysis of the active fractions showed the same pattern of amino acids, suggesting that the material was homogeneous.

Recent reports of purification of hypertensin from different species indicate pressor activity associated with larger numbers of amino acids on hydrolysis (Skeggs, Kahn & Shumway, 1954; Bumpus & Page, 1954; Kuether & Haney, 1955).

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The Pathway of Glutamic Acid Fermentation by *Clostridium tetanomorphum*. By B. A. FRY. (*Department of Microbiology, University of Sheffield*)

Glutamic acid is fermented by *Clostridium tetanomorphum* with the production of CO_2 (1 mole/mole amino acid), NH_3 and H_2, together with acetic and butyric acid (Woods & Clifton, 1938). In order to obtain more information concerning the fermentation pathway, washed cell suspensions of *Cl. tetanomorphum* were incubated anaerobically in Warburg manometers with glutamic acid labelled with ^{14}C in the α-COOH group. Carbon dioxide formed in the fermentation was absorbed in NaOH and later converted into $BaCO_3$ and assayed for ^{14}C. The volatile fatty acids were separated, and each estimated quantitatively and eventually obtained as the sodium salt, by a procedure involving partition chromatography on a column of celite buffered at pH 3·5 (Bueding & Yale, 1951). The total ^{14}C content of each fatty acid was determined by complete combustion to CO_2; the distribution of the isotope amongst the various C atoms of the fatty acid was determined by progressive degradation using the Schmidt reaction and oxidation of the resulting amine. Each C atom was thus obtained in the form of CO_2 which was absorbed in NaOH and converted into plates of $BaCO_3$ for the estimation of ^{14}C.

There was no ^{14}C in the CO_2 produced in the fermentation and, within experimental error, all the ^{14}C added to the system was recovered in the fatty acids. The results clearly showed that the CO_2 formed in the fermentation of glutamic acid is not derived from the α-COOH group of the amino acid. It is therefore probable that α-oxoglutarate is not an intermediate since the usual route by which this substance is metabolized involves decarboxylation

at the α-COOH group. Preliminary experiments indicate that in addition to a glutamic acid dehydrogenase, cell-free extracts contain another system which will bring about the anaerobic deamination of glutamic acid.

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Biosynthesis of Glycine and Serine in Normal and Folic Acid-Deficient Rats. By H. R. V. Arnstein. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Although glycine has long been known to be nutritionally dispensable, its metabolic origin remains obscure. Because of extensive metabolic interconversion of glycine and serine (Shemin, 1946; Sakami, 1948) either C_2 precursors of glycine or C_3 precursors of serine have been postulated (see Arnstein, 1954). The rate of glycine biosynthesis is, however, less than that of serine, which must therefore arise, at least partly, from sources other than glycine (Arnstein & Neuberger, 1953*b*).

In the present work, the biosynthesis of glycine and serine has been investigated in normal and folic acid-deficient rats, since in the latter the serine–glycine reaction is reduced (Elwyn & Sprinson, 1950). Uniformly labelled L-[^{14}C]alanine (0·59%) or D-[^{14}C]glucose (34%) were fed as part of a synthetic amino acid diet (diet AAD 2 described by Arnstein & Neuberger (1953*a*) except that glutamic acid was increased to 3·4%, aspartic acid omitted, homocystine replaced by methionine (1%) and [^{15}N]glycine fed at 0·1% level; [^{14}C]glucose, replacing cane sugar in diet AAD 2, was fed with 0·59% unlabelled L-alanine).

After 2 weeks the animals were killed and serine, glycine, alanine, glutamic acid and aspartic acid were isolated from the mixed visceral proteins. In all experiments, the radioactivities/C atom of the last three amino acids were similar. In normal rats, serine and glycine were equally labelled and had 70–90% of the radioactivity of the other amino acids when [^{14}C]glucose was fed, but only about 14% in the [^{14}C]alanine experiment. Glucose is therefore a better precursor of serine and glycine than is alanine or pyruvate, which arises from alanine by transamination.

In folic acid-deficient rats glycine biosynthesis, calculated from the dilution of the fed [^{15}N]glycine (cf. Arnstein & Neuberger, 1953*b*), was much reduced (98, 124, 129, 154·5 mg./day/100 g. body wt., compared with 185, 213 and 220·5 for normal animals). When [^{14}C]glucose was fed, the radioactivity of all the amino acids except glycine was substantially unchanged, but glycine had only about 50% of the radioactivity of serine. The incorporation of ^{15}N from glycine into serine, as well as into the other amino acids, was reduced to about 50% of the normal values, showing that the biosynthesis and catabolism of glycine are equally affected.

Glycine biosynthesis from glucose therefore probably involves serine as an intermediate, which is synthesized from a C_3 precursor, possibly a triose or triose phosphate, more closely related to glucose than to pyruvate.

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The Structure of the Mercapturic Acids formed in Rabbits from Trichlorobenzenes. By W. R. Jondorf, D. V. Parke and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

During a study of the metabolism of the isomeric trichlorobenzenes in the rabbit, we found that the 1:2:3- and 1:2:4-isomers gave rise to the excretion of small amounts (<1% of dose) of mercapturic acids (Jondorf, Parke & Williams, 1954). The amounts formed were too small for successful isolation. Attempts were therefore made to identify these acids, by hydrolysing the mercapturic acid fraction of the urine with alkali, separating the thiophenol formed by steam distillation and then identifying the trichlorothiophenols by oxidizing them to hexachlorodiphenyldisulphides, authentic samples of which had been synthesized.

From the mercapturic acid fraction of the urine of rabbits receiving 1:2:3-trichlorobenzene, 3:4:5:3′:4′:5′-*hexachlorodiphenyldisulphide*, m.p. and

mixed m.p. 151°, was isolated. The other possible isomer, 2:3:4:2′:3′:4′-*hexachlorodiphenyldisulphide* had m.p. 179° which was depressed by the urinary material. This suggests that 1:2:3-trichlorobenzene gives rise to 3:4:5-trichlorophenylmercapturic acid.

Similar experiments with 1:2:4-trichlorobenzene showed that two mercapturic acids were formed in roughly equal amounts. The trichlorothiophenol fraction isolated from the mercapturic acid fraction of the urine contained a liquid and solid which were separated mechanically. The liquid thiophenol yielded on oxidation, 2:4:5:2′:4′:5′-*hexachlorodiphenyldisulphide*, m.p. and mixed m.p. 136° and the solid, 2:3:5:2′:3′:5′-*hexachlorodiphenyldisulphide*, m.p. and mixed m.p. 168°. This suggests that 1:2:4-trichlorobenzene is metabolized to 2:4:5- and 2:3:5-trichlorophenylmercapturic acids.

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Studies on the Biosynthesis of Nicotinamide by Seed Embryos During Germination. By Chloe Klatzkin. (*Department of Agriculture, University of Leeds*)

Embryos of runner bean seeds, var. Scarlet Emperor, germinated under ordinary laboratory conditions have been found to contain free nicotinamide, as demonstrated by the method of Kodicek & Reddi (1951), it being produced during germination. Concurrent changes in the free amino acid contents of the bean embryos were also followed and some interesting changes and some unidentified amino acids observed. Embryos excised from similar beans and incubated at 22° on a synthetic medium containing inorganic salts, sucrose, ascorbic acid and thiamine, produced nicotinamide when either asparagine or tryptophan were added to the medium.

Treatment of the seeds with 1 in 1000 aqueous solution of mercuric chloride for 30 min. inhibited the formation of nicotinamide although the seeds remained viable and showed normal growth under aseptic conditions during the 12 days of the experiment. Embryos excised from treated beans and grown on the sterile synthetic medium did not produce any free nicotinamide with asparagine or tryptophan additions. These embryos were shown to be viable by measurements of their oxygen uptakes over a 25 hr. period.

Paper partition chromatograms were used to follow a number of changes in the effect of various amino acids added to the medium in which excised bean embryos were grown.

The work outlined indicates either the presence in the beans of a labile enzyme system responsible for the synthesis of nicotinamide or that there is, possibly, a symbiotic connexion of the embryo with micro-organisms upon which the seed is dependent for a supply of free nicotinamide.

This work was carried out during the tenure of a Junior Agricultural Research Fellowship held in the Department of Biochemistry, University College, London, and in the Department of Agriculture, Leeds University. I am glad to acknowledge help and fruitful suggestions from colleagues in both places.

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Adenosinetriphosphate-Guanidyl Phosphokinases in Annelid Worms. By G. E. Hobson (introduced by E. Baldwin). (*Department of Biochemistry, University College London*)

Following their isolation of taurocyamine from *Arenicola marina* and of glycocyamine from *Nereis diversicolor*, Roche, Thoai & Robin (1953*a*, *b*) made the suggestion that these guanidine bases might act as acceptors for labile phosphate groups in muscle contraction. The present work shows that the body-wall muscles of these Annelid worms contain enzymes capable of transferring phosphate groups to the respective bases. It seems probable that the resulting phosphorylated compounds correspond to the new 'arginine phosphate-like' phosphagen discovered in Annelid worms by Baldwin & Yudkin (1950). A 'creatine phosphate-like' phosphagen, probably creatine phosphate itself, was also discovered in certain Annelid species by the same authors. It has now been possible to demonstrate by paper chromatography the presence in muscle extracts of *Glycera gigantea* of substances identical in position and R_F value with creatine and creatine phosphate, thus confirming the tentative identification by Baldwin & Yudkin (Hobson & Rees, 1955).

It has further been shown by chromatographic methods that extracts of fresh muscle contain phosphotaurocyamine in the case of *A. marina* and phosphoglycocyamine with *N. diversicolor*. These

phosphagens were run using a strongly alkaline solvent to prevent hydrolysis. Hence a method has been evolved for chromatographing phosphagens directly, without previous hydrolysis and they can be detected by using Hanes & Isherwood's (1949) specific reagent for the phosphate group.

Finally, in order to find the optimum conditions for the transphosphorylation of taurocyamine and glycocyamine at the expense of adenosinetriphosphate (ATP) using enzymes from aqueous extracts of acetone powders of *A. marina* and *N. diversicolor*, the influence on the rate of reaction of the following factors have been studied: (i) magnesium concentration; (ii) the substrate specificity of the enzyme; (iii) pH of the reaction mixture; (iv) ATP concentration; (v) substrate concentration; (vi) enzyme concentration.

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An Unsaturated Bile Acid in the Chicken. By H. S. Wiggins. (*Postgraduate Medical School, London, W.* 12)

In the course of an investigation into the composition of the bile of 7-day-old chicks and adult hens, a new bile acid was isolated. The bile of the young chicks, taken immediately after death, was mixed at once with several volumes of ethanol. It was subsequently separated into glycine and taurine conjugated fractions (Wiggins, 1954). The taurine fraction was hydrolysed, the bile acids esterified with methanol and fractionated on silica-gel columns (Wootton, 1953). The methyl esters yielded a very small amount of a crystalline material eluted with the least polar solvent. A study of the infrared spectrum of this compound from 4000 to 800 cm.$^{-1}$ suggested the presence of two carbonyl groups, two double bonds and no hydroxyl groups. The position of three absorption bands (1668, 1621 and 1588 cm.$^{-1}$) agreed most closely with those attributed to a 3-oxo-4:6-diene (Jones & Herling, 1954). This was confirmed by the ultraviolet absorption spectrum which showed a peak at 284 mμ. (Sondheimer, Amendolla & Rosenkranz (1953) quote 283 mμ.).

Methyl 3-oxo-4:6-choladienate was therefore prepared by refluxing methyl 3β-hydroxychol-5-enate with manganese dioxide in benzene (Sondheimer, Amendolla & Rosenkranz, 1953). The ultraviolet spectrum of the synthetic compound from 210–350 mμ. showed one absorption peak at 283 mμ., $\epsilon_{max.}$ 4·4 in methanol. The infrared spectra of the synthetic and natural compounds were identical from 1800 to 1550 cm.$^{-1}$ and in the fingerprint region (1200–900 cm.$^{-1}$). The m.p. of the synthetic compound was 150–154°; of the isolated compound 150–152°; mixed m.p. 150–154°. This compound has been found in four pooled specimens of 7-day chick bile and one pooled specimen of hen bile. It forms about 0·5 % of the total bile acids of the taurine conjugated fraction. It is considered possible that this compound may be formed as an artifact since it would readily arise from a 7-hydroxy-3-oxo-4-ene.

The interest and encouragement of Dr I. D. P. Wootton and the advice of Dr W. Klyne is gratefully acknowledged.

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Observations on the Non-Collagen Constituents of Connective Tissue. By R. Bird and R. Consden. (*Special Unit for Juvenile Rheumatism, Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berks*)

In this communication we report some further observations concerning substances in connective tissue extracted by alkali (Consden, 1953; Consden & Bird, 1954). Human abdominal subcutaneous connective tissue, previously freed from fat and water-soluble material, was left in contact with N/5-KOH for 14 days at 0°. After this treatment the tissue still contained somewhat more than half of its original 2 % of carbohydrate. Little more carbohydrate or non-collagen protein was extracted by further alkali treatment, but increasing amounts of collagen were dissolved out. Part of the 14-day

extract was precipitated with acetic acid and the remainder with ethanol. The acetic acid-insoluble fraction formed viscous aqueous solutions at alkaline pH and contained about 10% carbohydrate (measured as hexosamine+hexose), whilst the ethanol fraction contained about 20%. On hydrolysis of these fractions, sulphate was liberated and only traces of phosphate. Paper electrophoresis indicated that each fraction contained a mixture of proteins (bromophenol blue staining) together with several acid polysaccharides (toluidine blue staining and metachromasia) some of which had higher mobilities than any of the proteins. Electrophoretic patterns were substantially the same after dialysis of the fractions. Most of the protein was not collagen; it contained tryptophan and was relatively rich in tyrosine and leucine. The total of the non-collagen protein extracted was calculated to be 3–4% of the original dry tissue and contained 4–5 times as much tyrosine as collagen. On mild hydrolysis of the fractions the sugars liberated were predominantly galactose, mannose and hexosamine. Some of the acid polysaccharides were separated by paper electrophoresis or by paper chromatography, followed by elution and hydrolysis and were shown to consist either of these three sugars or of galactose and hexosamine.

Although it is probable that the proteins and polysaccharides observed in these fractions were breakdown products of more complex material, the results suggest that in connective tissue, much of the tightly bound carbohydrate is a sulphated acid polysaccharide consisting of hexosamine and hexose, which may be associated with a tyrosine-rich protein.

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Antibody Formation in Slices of Granulomata Produced by Adjuvant. By B. A. Askonas and J. H. Humphrey. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The formation of antibodies has been studied in the granulomata produced in rabbits at the site of injection of Freund's adjuvant (Freund & McDermott, 1942) containing ovalbumin.

When the serum antibody reached a level of 6–10 mg. antibody protein per ml., the local cell mass was removed. Slices of this tissue were incubated at 37° for several hours in the presence of the rabbit's own serum, a 5% CO_2+O_2 gas mixture 2–4 μc [^{14}C]glycine and an isotonic medium (Raker, Taylor, Weller & Hastings, 1950) containing glucose and an amino acid mixture. After the incubation the tissue was homogenized in excess glycine and removed by high speed centrifugation. The antibody in the supernatant could be precipitated out specifically by addition of ovalbumin, and the radioactivity of the antibody-antigen complex determined by counting at infinite thickness. A γ-globulin fraction was then obtained by means of Na_2SO_4 using the method described by Kekwick (1940) for human γ-globulin. Although this fraction is not pure it contains mostly γ-globulin in the rabbit.

The slices of granulomata incorporated [^{14}C]-glycine rapidly into antiovalbumin as well as other γ-globulin and therefore contain cells that produce these proteins. The specificity of the incorporation of [^{14}C]glycine was tested by incubating the slices in the presence of rabbit serum containing anti-SSS III as well as antiovalbumin. The animal had been immunized against ovalbumin, and radioactivity was found only in the antibody the tissue was producing (i.e. antiovalbumin) and not in the antibody against SSS III. The incorporation of [^{14}C]-glycine therefore could not be due to non-specific adsorption or transfer of glycine. If the tissue is homogenized or frozen before slicing, no [^{14}C]-glycine is incorporated into the antibody and other γ-globulin.

The granulomatous tissue does not incorporate [^{14}C]glycine into the antibody as rapidly as the regional lymph nodes (10% of the rate). However, on the basis of total weight the cell mass appears to produce around 80% of the antibody in the rabbits confirming the conclusions of Humphrey & McFarlane (1955) using the whole animal.

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Incorporation of ^{14}C Amino Acids into Antibody and Other Serum Globulins *in vivo*. By J. H. HUMPHREY and A. S. MCFARLANE. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Incorporation of ^{14}C-labelled amino acids of *Chlorella* into plasma proteins and the subsequent decline of protein specific radioactivity, have been studied in normal and hyperimmunized rabbits. In animals immunized by intravenous injections of formalin-killed type III pneumococci, labelled antibody is detectable within 30 min., and peak labelling is reached in 4–5 hr., as happens with albumin and globulin in normal animals. The subsequent decline of specific radioactivity which is due both to replacement of labelled molecules by new unlabelled ones and to some re-utilization of the label, is also closely similar for albumin, 'normal' globulin and antibody, provided that the circulating mass of each protein remains constant. One month after immunizing rabbits by subcutaneous injection of ovalbumin or bovine serum albumin in Freund's adjuvant mixture, incorporation of radioactivity into albumin still occurs normally, but the serum antibody does not reach maximum specific activity for 50 hr. or more. The subsequent rate of disappearance of radioactivity in both cases is again similar to that in normal animals. The experimental animals carried large granulomatous masses of tissue at the antigen injection sites. Test injections of plasma labelled with ^{131}I given subcutaneously and also into these masses showed that maximum ^{131}I specific radioactivity was not reached in the plasma proteins until 40–50 hr. after injection. Thus the time at which a protein attains maximum specific radioactivity in the plasma is a useful indicator of the extra- or intra-vascular origin of the protein. So far as the latter is concerned there are facts to show that after intravenous injections of type III pneumococci, the main site of antibody production is in the lung.

The 'normal' globulin left behind after specific precipitation of antibody globulin attains nearly normal specific radioactivity at 4–5 hr. and thereafter this increases to a maximum at 50 hr. or more, indicating that the material is not homogeneous. It clearly consists partly of globulin produced in close connexion with the blood stream, as in normal animals, and partly of globulin originating in the granuloma. We were unable to find any evidence that the latter represented either precipitating or co-precipitating antibody against any of the constituents of the adjuvant mixture. It is concluded therefore that this protein is either an incomplete antibody to egg or bovine albumin or that it is globulin produced by cells in the granuloma but not arising from specific stimulation by the antigen.

Carbohydrate Metabolism in *Ascaris lumbricoides* from the Pig. By L. RATHBONE (introduced by E. BALDWIN). (*Department of Biochemistry, University College London*)

In suspensions of *Ascaris* muscle in 0·5 % potassium chloride solution fortified with ATP, Co I and Mg^{2+}, glucose and glycogen breakdown follow closely the Embden–Meyerhof scheme to the stage of phosphoglyceric acid. The latter accumulates in the reaction medium under the experimental conditions. Pyruvate must be added to the system before rapid glycolysis occurs and apparently reoxidizes the reduced DPN formed in the process (Rathbone & Rees, 1954).

A succinic oxidase system with unusual properties was found to be present in a particulate fraction isolated from the muscle of *A. lumbricoides*. The rate of uptake of oxygen increased with increasing oxygen tension and in an atmosphere of pure oxygen the rate averaged 240 μl./mg. N/hr. at 38°.

The respiratory end system appeared to be atypical. It was not possible to demonstrate the presence of a typical cytochrome oxidase. Hydrogen peroxide was formed in the oxidation of succinate. Addition of methylene blue to the succinic oxidase system caused a relatively great increase in Q_{O_2}. DPN and ethylenediaminetetraacetate brought about further increases.

α-Oxoglutarate, L-malate and fumarate stimulated the uptake of oxygen when added to fortified particulate suspensions from muscle and addition of methylene blue greatly increased Q_{O_2}. Citrate and *cis*-aconitate were without effect on the system.

Chin & Bueding (1954) claimed that the perienteric fluid of *Ascaris* contains the substrate and also a heat-stable, non-dialysable factor necessary for oxidative phosphorylation. In the present study oxidative phosphorylation was found to occur in fortified particulate preparations from muscle in the presence of perienteric fluid.

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DEMONSTRATIONS

Studies on the Salivary Sodium/Potassium Ratio in Man. By G. L. S. Pawan. (*Medical Unit, Middlesex Hospital, London, W. 1*)

Salivary specimens were obtained by a standard technique modified from Frawley & Thorn (1951). Sodium and potassium were estimated by an EEL flame photometer.

A marked diurnal variation in the ratio, due mainly to alteration in sodium concentration was found in 20 normal adults studied. Highest values were found in the morning on waking, falling to minimum values around midday, rising slowly in the afternoon and more rapidly in the evening. Two subjects with Addison's disease failed to show a diurnal rhythm. Four normal subjects after night-work for two months (sleeping in daytime) showed an altered diurnal rhythm.

The sodium/potassium ratio varied with rate of flow of saliva, but it was usually possible, by this method, to obtain fairly reproducible rates of flow and ratios in each subject, when specimens were collected at the same time of day, preferably in the afternoon. Values of the ratio in 230 normal adults were in the range 0·15–2·10 (mean 0·58). In three patients with Cushing's syndrome values were below 0·15, rising after adrenalectomy and maintenance on cortisone above 0·4. In two patients with Addison's disease (untreated) values were above 4·5, falling during adrenocortical therapy below 2·0.

Significant reductions in the ratio within 3 hr. were found in five normal males after 20–80 μg. oral aldosterone, and in four normal subjects after 100–200 μg. oral fluorohydrocortisone. Aldosterone (100 μg./day orally) reduced the ratio in one case of Addison's disease studied. Single oral doses of hydrocortisone (up to 20 mg.) or cortisone (up to 100 mg.) produced no significant reduction in the ratio of five normal subjects. In a patient with Addison's disease, the ratio was reduced by the daily administration of 100 mg. oral cortisone acetate or of 5 mg. DOCA intramuscularly. ACTH (25 mg. in 5 ml. saline) injected intramuscularly did not lower the ratio in 15 normal males.

Using the standard collection technique, the ratio was unaffected by ingestion of ordinary meals, water (1 litre), sodium chloride (15 g.) or potassium chloride (10 g.).

Some normal subjects show occasional anomalous changes in the ratio unrelated to rate of flow of saliva. The effect of physical exercise, surgical operation, and winter–summer temperature changes were variable.

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The Location of Substances on Paper Chromatograms by Differential Charring and the Use of an *n*-Propanol–Water Solvent Containing an Acetic Acid–Sodium Acetate Buffer for the Chromatography of Phosphate Esters. By P. C. Caldwell. (*Department of Biophysics, University College London*)

A number of substances which are difficult to locate because there is no simple spraying agent for them can be detected by a process of differential charring. The chromatogram is held over a heated surface so that it gradually starts to darken. It is found that many of the regions in which substances are present tend to char more rapidly than the rest of the paper and hence appear as dark spots. The method, which can only be applied successfully to chromatograms run in solvents which can afterwards be volatilized, is particularly useful for detecting ions. The demonstration will show its use in the location of isethionate (R_F 0·70), which is probably the principle anion of squid axoplasm (Koechlin, 1954), ethyl hydrogen sulphate (R_F 0·80) and sulphate (R_F 0·32) after their separation in the *n*-propanol–ammonia solvent of Hanes & Isherwood (1949). Potassium (R_F 0·33) moves independently of the anions and is also detected. The method can also be used to detect ions after ionophoresis if this is done in the presence of a salt which can afterwards be volatilized, such as ammonium formate.

The demonstration will also include chromatograms of phosphate esters run for 12–15 hr. in a solvent of 70 ml. *n*-propanol and 30 ml. of a 2 M acetate buffer of pH 4. The esters were located by means of the molybdate reagent of Hanes & Isherwood. The relative distances travelled by some important phosphate esters are: Orthophosphate 1·00; ATP 0·19; ADP 0·42; AMP 0·91;

Fructose 1:6-diphosphate 0·26; Fructose 6-phosphate 0·84; Creatine phosphate 0·48. The merit of this solvent is its ability to separate creatine phosphate from AMP and fructose 6-phosphate, a separation not possible with solvents tried previously (Caldwell, 1953).

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Borate Buffer as a Medium for the Ionophoresis of Human Serum Proteins. By E. Abdel Wahab, G. Adjutantis and D. J. R. Laurence. (*Postgraduate Medical School of London, Ducane Road, W.* 12)

Borate buffer was used by Consden & Stanier (1952) to obtain separations by ionophoresis of mixtures of monosaccharides and by Adjutantis (1954*a*, *b*) for resolution of cytoplasmic proteins of rat liver. Martin & Franglen (1954) have described an effect of borate on the α- and β-globulins of human serum.

A number of examples are shown of serum protein samples separated in veronal and in borate buffers (pH 8·6). The α_2- and β-globulins which appear in the veronal buffer are each resolved in the borate buffer into a pair of distinct bands and quantitative data supporting this view are given. The albumin/globulin ratio is unaffected by change from a veronal to a borate buffer.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **339th Meeting** *of the Biochemical Society was held in the Department of Biochemistry of the University of Liverpool on Friday, 15 April 1955 at 11 a.m. when the following papers were read:*

COMMUNICATIONS

The Hydroxylation of Coumarin and the Cyclization of *o*-Coumaric Acid *in vivo*. By J. A. R. Mead, J. N. Smith and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

When coumarin is fed to rabbits, paper chromatography of various fractions of the urine shows that 3- and 7-hydroxycoumarins and *o*-coumaric acid (*o*-hydroxy-*trans*-cinnamic acid) are excreted. The presence of 4-hydroxycoumarin is also suspected but salicylic acid is absent. Hydroxylation in the 7-position of the coumarin ring of the anticoagulant, Tromexan, has also been shown to occur in man by Burns, Weiner & Brodie (1953) and Burns, Wexler & Brodie (1953).

Coumarin is the lactone of *o*-hydroxy-*cis*-cinnamic acid (coumarinic acid) and its conversion to *o*-coumaric acid involves a *cis* to *trans* change. It was therefore of particular interest to find out when the reverse change, *trans* to *cis*, occurred *in vivo*. It was first shown that *o*-coumaric acid (pK_a 4·6) (dose 0·2 g./kg.) was only conjugated to a low degree with glucuronic acid (9–17 %) and probably not at all with sulphuric acid (0–2 %). Unchanged *o*-coumaric acid could be isolated from the urine. From the glucuronide fraction of the urine, two glucuronides were isolated. The major one was the stable, non-reducing, ether-glucuronide of *o*-coumaric acid (*o-glucuronosido*-trans-*cinnamic acid*, m.p. 222°, $[\alpha]_D^{18} - 81{\cdot}3°$ in 0·1N-NaOH; Found: C, 51·6; H, 4·8. $C_{15}H_{16}O_9$, $0{\cdot}5H_2O$ requires C, 51·6; H, 4·9 %). The second, which reduced hot alkaline copper reagents, was identical with 4-hydroxycoumarin glucuronide (m.p. and mixed m.p. 184–185°) (cf. Roseman, Huebner, Pancratz & Link, 1954). The isolation of this latter glucuronide shows that *o*-coumaric acid can be converted into coumarin derivatives *in vivo*, a reaction which involves a change in configuration followed by cyclization. The mechanism of this reaction will be discussed.

The glucuronides of 3- and 4-hydroxycoumarins have been previously described (Flatow, 1910; Roseman *et al.* 1954). The latter is a reducing enol-glycoside whereas the former is not. According to the views of Anderton, Smith & Williams (1948), hydroxyl groups with pK_a's <7 do not conjugate with sulphate. 4-Hydroxycoumarin (pK_a 5·8) forms only a glucuronide in the rabbit, whereas 3-hydroxycoumarin conjugates with both glucuronic and sulphuric acid.

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A New Free Amino Acid in Cabbage. By R. L. M. Synge and J. C. Wood. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

In studying 'bound amino acids' in plant juices we noticed that diffusates (cf. Synge, 1951) from cabbages gave, after 24 hr. in 6N-HCl at 105°, a greatly increased spot at the valine position on phenol–NH_3/'collidine' paper chromatograms. The parent substance was isolated by adsorption from the original diffusate on to a column of Zeo-Karb 215 followed by displacement with NH_3 (cf. Westall, 1950). The substance emerged with aspartic acid, glutamic acid etc. It was separated from these on a phenol–water kieselguhr partition chromatogram (aqueous phase stationary), from which it emerged first. After evaporation, crystallization from aqueous ethanol yielded a product accounting for 4·2 % of the N of the diffusate, 2·2 % of total N and 0·56 % of dry matter of the cabbage. M.p. 174–5° (decomp.); $[\alpha]_D^{23} + 118°$ (in water, $c = 1{\cdot}5$); $[\alpha]_D^{24}$, $+168°$ (in N-HCl, $c = 1{\cdot}5$). Empirical formula, $C_4H_9O_3NS$. Behaviour on electrophoresis in acid and neutral solution and on potentiometric titration

indicated an ampholyte having pK_1 1·7, pK_2 8·0. Amino N (HNO_2) was 96 %, carboxyl N (ninhydrin-CO_2) 90 % of total N.

We consider that the new amino acid is one of the diastereoisomeric forms of L-*S*-methylcysteine sulphoxide. A synthesized mixture of the diastereoisomers (cf. Bentley, McDermott & Whitehead, 1951) coincided with it on paper chromatograms developed as above and coloured with ninhydrin. On treatment with hot HCl as above, both synthetic and natural materials were completely destroyed, giving 0·4–0·5 mol. prop. of amino acid identified on paper chromatograms as *S*-methylcysteine (coincident with valine but destroyed by Br_2). The rest of the N was converted to NH_3; keto acid was also found (cf. Vilkki, 1954). On treatment with H_2O_2-molybdate followed by paper chromatography, synthetic and natural materials both showed very incomplete conversion to a new ninhydrin-staining compound, judged to be the sulphone, which coincided in position with a spot obtained after treatment of *S*-methylcysteine in the same way. The relations of sulphoxide and sulphone spots to that of *S*-methylcysteine were similar to those for corresponding methionine derivatives (Dent, 1948).

The unexpectedly low carboxyl p*K* of the new compound presumably determines behaviour on the ion-exchange resin. Methionine sulphoxides behave similarly (Moore & Stein, 1954).

The new compound seems to account for a substantial proportion of the organic S of cabbage (Dahlen, 1874).

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Metabolism of Carotenoids in the Rat: Lycopene. By R. H. Painter and J. Glover. (*Department of Biochemistry, University of Liverpool*)

It was suggested by Glover & Redfearn (1954) that the transformation of β-carotene into vitamin A *in vivo* involved terminal oxidation rather than central fission. It was of interest to know if lycopene could be broken down like β-carotene to yield apo-15-lycopenol, isomeric with vitamin A (β-apo-15-carotenol). Meunier, Jouanneteau & Zwingelstein (1950) reported that an oxidation product of lycopene was vitamin A active and had similar properties to retinal$_2$. Since retinal$_2$ is now known to possess the safranal-type ring structure (Farrar, Hamlet, Henbest & Jones, 1952), these observations have not been satisfactorily explained.

Crystalline lycopene ($E^{1\%}_{1cm.}$ (471 mμ. in light petroleum) = 3530; m.p. 167°), was oxidized with H_2O_2 in the presence of OsO_4 (Wendler, Rosenblum & Tishler, 1950). Apo-lycopenals with 9, 7 and 5 ethylenic bonds respectively, conjugated with the aldehyde group were isolated. The substance apo-15-lycopenal with 5 ethylenic bonds has $\lambda_{max.}$ at 383 mμ. in light petroleum and at 408 mμ. in chloroform (cf. retinal$_2$: 385 and 407 mμ.). The antimony trichloride reaction product however, has $\lambda_{max.}$ at 660 mμ. (retinal$_2$ chromogen: $\lambda_{max.} = 735 \rightarrow 705$ mμ.). The alcohol obtained by $LiAlH_4$ reduction, has three absorption maxima at 327, 342 and 360 mμ. in light petroleum. The antimony trichloride blue colour, has $\lambda_{max.}$ at 620 mμ.

The apo-15-lycopenal obtained chemically and the lycopene were administered to separate groups of vitamin A-deficient rats at dose levels of 15 and 100 μg./day respectively over a period of 12 days. No growth response was observed in either group whereas vitamin A-fed (2 μg./day) controls commenced growing within a few days. At the end of the experiment, no apo-15-lycopenol was recovered.

The livers of animals given lycopene retained 15 % of the total fed. Later, the livers and intestines of two vitamin A-deficient animals which had been given single doses of lycopene (0·6 mg.) and apo-12′-lycopenal (0·65 mg.) were examined separately for metabolites 4 hr. after dosing. Traces of apo-15-lycopenol were detected in both intestines but not in the livers. Other oxidation products giving a deep red colour with $SbCl_3$ were detected in the intestine of the lycopene-fed animal.

The conclusions reached are (i) that lycopene is readily metabolized and (ii) that none of the oxidations products possess vitamin A activity. In fact, the ultraviolet spectrum of apo-15-lycopenol and its colour test show that it is completely different from vitamin A_2.

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The Carotenoids of *Chlorobium* spp. By T. W. Goodwin and D. G. Land. (*Department of Biochemistry, University of Liverpool*)

Little is known of the carotenoids in green sulphur bacteria. In early experiments, van Niel & Muller (1931) failed to detect them in their cultures but, later Katz & Wassink (1939) reported their presence in a different strain. Three *Chlorobium* spp. have now been examined: *C. thiosulphatophilum* (from Dr June Lascelles) *C. limicola* and a halophilic species (from Mr K. R. Butlin). In all species the major pigment has been identified as γ-carotene; it accounts for about 80% of the total carotenoids present. The other main constituent is almost certainly rubixanthin (3-hydroxy-γ-carotene); all its properties are identical with those of rubixanthin but final confirmation by comparison with an authentic specimen of rubixanthin has not yet been possible. A number of other pigment fractions were observed in trace amounts; they have very indefinite absorption spectra and are probably oxidative artifacts.

Diphenylamine specifically inhibits carotenogenesis in a number of organisms. At a concentration of 1/140 000, it inhibits carotenoid synthesis almost completely in all three *Chlorobium* spp. Bacteriochlorophyll synthesis is also inhibited somewhat, but not to such a marked extent.

Phytoene, phytofluene, ζ-carotene etc. accumulate in diphenylamine cultures of *Phycomyces blakesleeanus* and *Rhodospirillum rubrum* (Goodwin, 1952; Goodwin & Osman, 1954); only traces of these polyenes are produced by diphenylamine cultures of *Chlorobium* spp. Other, as yet unidentified, colourless fluorescent compounds do, however, accumulate.

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The Occurrence of 6-Methylaminopurine in Microbial Deoxyribonucleic Acids. By D. B. Dunn and J. D. Smith. (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

6-Methylaminopurine was first isolated from the deoxyribonucleic acid (DNA) of the thymine-requiring *Bacterium coli* 15T$^-$ grown under conditions of thymine deficiency (Dunn & Smith, 1955). We subsequently showed that DNA from this bacterium grown on excess thymine contained small quantities of the base.

To demonstrate the presence of the base in other bacterial DNA's we have used a 0·5 hr. hydrolysis in N-HCl at 55° to liberate the purines, followed by two-dimensional paper chromatography in *iso*propanol–water–HCl (Wyatt, 1951) and *iso*propanol–water–NH_3 (Markham & Smith, 1952). In DNA's from *Bact. coli* B, *Bact. coli* K_{12} and *Aerobacter aerogenes* the base is present in amounts equivalent to about 2% of the adenine, while in *Mycobacterium tuberculosis* and T_2r^+ bacteriophage it amounts to about 0·4% of the adenine. We have failed to detect the base in ribonucleic acid from *Bact. coli* 15T$^-$ or in DNA from horse spleen, wheat germ or yeast.

A comparison of the bases in *Bact. coli* 15T$^-$ DNA grown in conditions of excess thymine and of thymine deficiency suggested 6-methylaminopurine replaces thymine. This is difficult to fit into the model for DNA suggested by Watson & Crick (1953), but as we find nucleic acid containing large quantities of the base is less readily precipitated by acid and ethanol than is normal nucleic acid, this may indicate that it is of an abnormal type. Our analyses of *Bact. coli* B DNA moreover, in agreement with earlier analyses (Smith & Wyatt, 1951), are consistent with the suggestion that in this DNA, the new base replaces adenine residues.

Synthetic 6-methylaminopurine was found to inhibit the growth of *Bact. coli* 15T$^-$. This effect was reversed by adenine and not by thymine. *Bact. coli* 15T$^-$ grown on a synthetic medium containing 0·0005 M 6-methylaminopurine showed no significant increase in the amount of the base in the DNA. The bacterial growth rate was reduced immediately on addition of the 6-methylaminopurine in contrast to the delay of 1–2 hr. before the onset of inhibition observed with 8-azaguanine (Matthews & Smith, 1954) and 5-halogenated uracils (Dunn & Smith, 1954) which are incorporated into nucleic acids.

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The Vitamin A-Deficiency Syndrome in the Rat. By F. W. Heaton, J. S. Lowe and R. A. Morton. (*Department of Biochemistry, University of Liverpool*)

The accepted signs of vitamin A deficiency are (1) defective scotopic vision; (2) retarded growth, leading to loss of weight; (3) hyperkeratosis including xerophthalmia; (4) skeletal abnormalities and (5) disturbance of normal sexual processes.

Experiments on vitamin A-deficient rats have been undertaken in order to investigate how the unsaponifiable constituents of different organs differ in nature and quantity from those of the corresponding unsaponifiable fractions of control and stock rats.

Fatty livers occurred in 10 out of 180 A-avitaminotic rats, distended stomachs in 7, distended bladders in 29, but infected submaxillary glands (often observed in avitaminosis A) were seen only in 3 cases. Fatty livers have previously been noted in vitamin A-deficient rats (Monaghan, 1933) and Mayer & Krehl (1948) have published photographs of grossly distended stomachs.

The unsaponifiable fractions from the different organs were chromatographed on weakened alumina (Brockmann, Grade III). A new series of constituents was observed, each member possessing its typical ultraviolet absorption. 'SA'-characterized by $\lambda_{max.}$ 272 mμ., $\lambda_{min.}$ 235 mμ., inflexions 330 and 410 mμ.; 'SC' $\lambda\lambda_{max.}$ 275 mμ., 330 mμ. and inflexions 233 and 283 mμ. (*cyclo*hexane). These compounds have been previously reported (Lowe, Morton & Harrison, 1953).

Both SA and SC are normal, but very minor constituents of rat liver, kidney and intestine. In vitamin A deficiency, however, the amount of SC in liver greatly increases, liver from xerophthalmic rats containing approximately 90 times as much SC as liver from stock rats and liver from rats on moderate intakes (25 i.u./day) containing about forty times as much. The amount of SC in intestine is also slightly greater for vitamin A-deficient rats than for stock rats while the amounts in the kidney appear to be unchanged. The amounts of SA show an increase in the vitamin A-deficient rat liver. The 7-dehydrosteroid content of intestine from vitamin A-deficient rats shows a fall when compared with control and stock animals, whilst in vaginas, bladders and submaxillary glands a reversed effect is noted.

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Unsaponifiable Fractions from Kidneys and Livers of Various Species. By N. F. Cunningham, J. S. Lowe, L. Mervyn, R. A. Morton and J. Vernon. (*Department of Biochemistry, University of Liverpool*)

Heaton, Lowe & Morton (1955) have described the occurrence of two substances 'SA' ($\lambda_{max.}$ 272 mμ. $\lambda_{min.}$ 235 mμ.; inflexions 330 and 410 mμ. *cyclo*hexane) and 'SC' ($\lambda\lambda_{max.}$ 275 mμ. and 330 mμ., inflexions at 233 and 283 mμ. *cyclo*hexane) in liver and kidney of both normal and vitamin A-deficient rats. The question arises whether these metabolites are specific for the rat or whether they occur in other animals.

A survey of the unsaponifiable fractions of liver and kidney from pig, sheep, dog and pullet is being made together with a parallel study on human kidney. In the course of these investigations a further new minor constituent of kidney has emerged; 'SE', possessing $\lambda_{max.}$ 290 mμ.; $\lambda_{min.}$ 255 mμ. inflexion 300 mμ. *cyclo*hexane.

SA and SC have been detected in the liver and kidney of pig, sheep, dog and pullets while both are present in the human kidney.

So far, the richest source of SA appears to be hen liver, while human kidneys contain the highest concentration of SC.

SE has been detected in cat, dog, sheep and human kidney.

It is clear that SA and SC are minor constituents of liver and kidney for the various species studied but SE seems not to be so widespread.

The distribution of these substances in normal and abnormal conditions is being studied. The human postmortem material so far examined does not allow a picture of the normal to be presented. With that exception, all the information refers to animals showing no signs of abnormality.

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Portal Blood Analysis in the Study of the Absorption of Ruminal Fermentation Products. By E. F. Annison, K. J. Hill and D. Lewis. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

Schambye & Phillipson (1949) and Schambye (1951) have shown that the volatile fatty acids (VFA's) produced as a result of ruminal fermentation in the sheep are absorbed into the portal blood, but complete analyses of the VFA's and other absorbed fermentation products are not available. We have carried out a number of preliminary experiments in sheep in which a wide-bore polythene catheter was passed through the substance of the caudate lobe of the liver and then via the portal branch of that lobe into the portal vein. The catheter was further anchored in position with plastic sponge (Grindlay & Waugh, 1951). After 7–14 days difficulty was experienced with clot formation in the catheter, but the method has enabled us to carry out a number of experiments where large volumes (20–25 ml.) of portal blood were required at frequent intervals.

The experiments were concerned with the concentrations of VFA's, ammonia and ketone bodies in portal and peripheral blood, determined by the procedures of Annison (1954), Conway & Cooke (1939) and Greenberg & Lester (1944) respectively: rumen contents were examined at the same time. When the animals had been fasted 24 hr. the concentrations of VFA's, ammonia and ketone bodies in portal blood and rumen contents were relatively constant. After feeding chopped hay or treated straw with supplements of starch or casein the changes in rumen VFA's and ammonia were roughly paralleled by those in portal blood. Thus after a sheep had eaten 700 g. of a ration rich in starch and casein hydrolysate the total VFA's in the rumen rose from 45 to 94 m-moles/l. in 2 hr. as compared with an increase from 1·31 to 1·78 m-moles/l. in portal blood. Complete analysis of the VFA's in each sample of portal blood and rumen contents by gas-liquid partition chromatography suggested that the concentration of butyrate in portal blood was proportionally smaller than in rumen contents. Similarly, after eating a ration rich in casein, the ammonia concentration in portal blood increased from 0·3 to 0·9 m-mole/l. in 5 hr. and in the same period ammonia in rumen contents increased from 15 to 60 m-moles/l. During this time the concentration of ammonia in jugular blood was very low and constant. A higher concentration of ketone bodies was observed in portal blood than in peripheral blood.

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Uridine-diphosphate-glucuronic Acid and Ester Glucuronide Synthesis. By G. J. Dutton. (*Department of Biochemistry, Queen's College, Dundee, University of St Andrews*)

Two kinds of glucuronic acid conjugate are known, depending on the aglycone; alcohols and phenols give rise to glycosidic ('ether') linkage, and certain carboxylic acids to acylal ('ester') type (see Teague, 1954).

The factor responsible for synthesis, in liver homogenates, of *o*-aminophenyl- and (−)-menthyl-glucuronides (Dutton & Storey, 1951, 1954) was shown to be uridine-diphosphate-glucuronic acid (Dutton & Storey, 1953; Storey & Dutton, 1955). The present communication extends these observations to the formation of ester-linked glucuronides.

$1{\cdot}4 \times 10^{-4}$M-Anthranilic acid replaced the *o*-aminophenol–ascorbic acid mixture in the homogenate system (Dutton & Storey, 1954); measurement of glucuronide synthesis was by small-scale modification of the method of Shirai & Ohkubo (1954), wherein anthranilic acid is liberated by mild alkaline hydrolysis from its (ester) glucuronide. Presence of the factor during incubation was essential for the formation of an ether-insoluble conjugate which liberated anthranilic acid under these conditions. *o*-Aminophenyl-glucuronide caused no interference, and Shirai & Ohkubo (1954) found none from aniline, acetanilide, 5-hydroxyanthranilic and *N*-acetylanthranilic acids. Since *o*-aminobenzoyl-glucuronide has not been obtained pure, the *p*-isomer (see Bray, Lake, Neale, Thorpe & Wood, 1948) was also used, and similar results obtained. Paper chromatography of the conjugate gave a spot at the known *p*-aminobenzoyl glucuronide (PABG) level (spraying with diazotization reagents), and the ether-soluble product of alkaline hydrolysis travelled like known *p*-aminobenzoic acid.

Confirmatory evidence that the conjugate was a glucuronide came from the use of β-glucuronidase. As recently reported (Levvy & Worgan, 1955), it was found that this enzyme could hydrolyse ester glucuronides (here PABG) with strong inhibition from boiled saccharate solution (see Levvy, 1952;

Levvy & Worgan, 1955). β-Glucuronidase liberated, from the unknown conjugates, ether-soluble compounds behaving colorimetrically and chromatographically like the respective free acids. Such liberation was 80–90% inhibited by 0·00012M boiled saccharate. β-Glucuronidase esterase activity is apparently limited to ester glucuronides (Levvy & Worgan, 1955).

Ester-type conjugation did not appear to require conditions of pH or oxygen supply different from those previously needed (Dutton & Storey, 1954). That the same enzyme system might be involved was suggested by inhibition of *o*-aminophenylglucuronide synthesis by added benzoate, which was lessened by increasing *o*-aminophenol concentration.

Crude factor (Dutton & Storey, 1954) was used in preliminary experiments; confirmation was obtained with chromatographically pure preparations.

The PABG was obtained from an equimolecular complex of *p*-aminobenzoic acid and PABG, and also recrystallized from a cruder PABG specimen, both prepared 9 years ago and very kindly given by Dr W. V. Thorpe. I thank the M.R.C. for a grant.

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Photo-activation as a Factor in the Cephalin Cholesterol Reaction. By O. Bassir and J. Hall.
(*Department of Biochemistry, Westwood Hospital, Beverley*)

The dynamics of the mechanism of action of Hanger's (1939) cephalin cholesterol test of liver function has not been completely elucidated. Neefe & Reinhold (1944) experimented with sera of thirteen normal persons and seven patients with liver disease, and showed that exposure of the serum-antigen suspension to bright light increased the flocculation.

However, no quantitative analysis of this phenomenon appears hitherto to have been recorded.

In the present work, over 200 different specimens of blood serum from hospital patients were examined. Duplicate experiments were performed (*a*) in darkness, and (*b*) in diffused light. The serum–saline–antigen mixtures were left standing in stoppered glass tubes, and flocculation readings (1, slightest; 4, complete) taken after 24 and 48 hr., respectively. Control tubes were set up simultaneously. The batch of cephalin cholesterol antigen used was obtained from Difco.

The results obtained with specimens exposed to light are designated L; the others D.

It was found that for values of $D=0, 1, 2, 3$, respectively, the percentage incidence of $(L-D)>0$ was 65, 75, 30, 16, in 24 hr.; and 81, 85, 65, 6, in 48 hr. This indicates the extent and manner in which photo-activation influences the results of the tests.

The sera studied could be divided into two groups: (*a*) (expected positives) those from patients suffering from hepatic disease; (*b*) (expected negatives) those from patients with no clinical evidence of liver dysfunction (Hanger, 1939). When our 24 hr. results were analysed in this way for $D=0, 1, 2, 3, 4$, respectively, it was found that the percentages of the total number of tests in which $(L-D)>0$ were 24, 41, 18, 3 for the expected negatives; and 24, 33, 23, 9 for the expected positives. This suggests that the photosensitivity of the two kinds of blood serum is of the same order, and not the main cause of the insensitiveness of the test as far as differential diagnosis is concerned. The last deduction was confirmed by correlating the results of the dark experiments at 24 and 48 hr. In this case, for 24 hr. values of 0, 1, 2, 3, respectively, $(48-24)>0$ occurred in 16, 22, 6 and 1% of the total number of expected negatives; and 10, 25, 13 and 5% of the total number of expected positives.

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The Estimation of Formaldehyde by Schryver's Method. By C. M. Dowse and J. A. Saunders. (*Department of Physiology, The Medical School, King's College, Newcastle upon Tyne* 1)

Schryver (1910) reported a method for estimating formaldehyde using a reaction with phenylhydrazine hydrochloride. The product when oxidized with potassium ferricyanide and acidified with hydrochloric acid gives a red colour. Various authors (Callow, 1927; Ramsay & Stewart, 1941; Desnuelle & Naudet, 1945) have used this method and all use slightly different conditions. We have investigated the reaction and find that the pH of the solution and the time during which formaldehyde and phenylhydrazine react are important. We found that the addition of acidified ferricyanide gave more consistent results than adding ferricyanide followed by acid.

The procedure adopted is to use 2 ml. solution ($<45\,\mu$g. formaldehyde) and add 2 ml. 1% (w/v) phenylhydrazine hydrochloride in 0·1 M citrate buffer pH 3·5. After standing 30 min. add, with shaking, 7 ml. acid ferricyanide (5 ml. conc. HCl: 2 ml. 2% (w/v) potassium ferricyanide) and dilute with water to 25 ml. Measure the extinction at once on an absorptiometer. The phenylhydrazine (AR) should be recrystallized from hydrochloric acid. The ferricyanide (AR) is recrystallized from water. The phenylhydrazine solution and the acidified ferricyanide solution are made up freshly for each batch of determinations.

With amounts of formaldehyde less than 45 μg. a straight line was obtained passing through the origin, but with amounts greater than 45 μg. proportionately less colour was formed. The standard deviation of 172 determinations was 1·52 which is equivalent to 1·25 μg. of formaldehyde. The mean of three replicate determinations will have 5% confidence limits of $\pm 1{\cdot}44\,\mu$g. formaldehyde.

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A Threonine-Isoleucine Relationship in *Bacillus subtilis*. By Cynthia E. Sewell and H. K. King. (*Biochemistry Department, Johnston Laboratory, University of Liverpool*)

Experiments with auxotrophic mutants of *Neurospora crassa*, *Bacillus subtilis*, and *Escherichia coli* suggest that threonine and isoleucine may arise from a common precursor (Adelberg, 1951). Experiments with a 'wild type' (prototrophic) *B. subtilis* support this idea. The organism may be grown on a simple synthetic medium but washed suspensions exhibited poor activity in the synthesis of alanine from pyruvate by reductive amination (Fairhurst, King & Sewell, 1952). This system is highly active in organisms grown on nutrient broth or casein hydrolysate. A mixture of 18 amino acids yielded fully active suspensions: no loss in activity resulted when either threonine or isoleucine was omitted, but with the omission of both the activity fell to the value obtained on the minimal medium. For maximal activity other amino acids are also needed and these requirements are now being worked out.

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The Oxidases of *Polystictus versicolor* and *Neurospora crassa* in Relation to their Endogenous Respiration. By D. Boulter (introduced by T. W. Goodwin). (*Hartley Botanical Laboratories, University of Liverpool*)

It has been suggested by several authors (see James, 1953) that polyphenol oxidase is the terminal oxidase of the normal respiration of some higher plants. Since fungi are a rich source of polyphenol oxidase enzymes it was of interest to investigate this possibility with respect to fungal respiration.

The endogenous respiration of *Polystictus* and *Neurospora* was measured as O_2 uptake in Warburg manometers by the direct method (Dixon, 1943) both in the presence and absence of respiratory inhibitors, viz. potassium cyanide, carbon monoxide (in the light and in the dark), sodium diethyldithio-

carbamate and salicylaldoxime. These inhibitors were shown to be in part selective in their action on cytochrome oxidase and polyphenol oxidase extracted from the mycelium.

Results of such experiments taken together with the results from experiments with cell-free extracts indicated that the O_2 uptake of the mycelium of these fungi was mediated mainly, if not completely, via a cytochrome *c*–cytochrome oxidase system. Polyphenol oxidase, although known to be present, appeared to play no signnificant part in the endogenous respiration.

In view of these findings, a spectroscopic examination of fungi from all major taxonomic groups for the presence of cytochromes has been made, using Keilin and Hartree's low temperature technique (Keilin & Hartree, 1949). Of the forty fungi investigated, all but four showed typical absorption bands closely corresponding to the alpha absorption bands of the reduced cytochromes, as seen in yeast suspensions. In the four cases where such bands were not seen, pigmentation obscured observation.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **340th Meeting** *of the Biochemical Society was held in the University of Reading on 14th May 1955 at 11 a.m. when the following papers were read:*

COMMUNICATIONS

Biotin and Tryptophan Metabolism in the Rat. By C. E. Dalgliesh. (*Postgraduate Medical School, Ducane Road, London, W. 12*)

Biological transformation of tryptophan into nicotinic acid involves several enzymic steps in which B vitamins participate. By studying the excretion of metabolites after giving tryptophan to vitamin-deficient rats, it is possible to establish the stage in tryptophan metabolism at which the vitamin functions. Such experiments with pyridoxin (Dalgliesh, 1952), riboflavin (Charconnet-Harding, Dalgliesh & Neuberger, 1953) and thiamine (Dalgliesh, 1954) have previously been reported. Recently Shanmuga Sundaram, Tirunarayanan & Sarma (1954) claimed that in *Neurospora* biotin is concerned in the first stage of tryptophan metabolism, in which formylkynurenine is formed. In an (unpublished) survey of B vitamins the author had found no evidence for such a relationship in the rat. The problem has therefore been reinvestigated.

Three groups of young male white rats, control, biotin-deficient and biotin-pyridoxin-deficient, were fed a diet based on dried egg-white (containing avidin) as protein source. Succinylsulphathiazole (1% by weight) was added to inhibit bacterial synthesis of biotin in the gut. Appropriate vitamin mixtures were given daily by stomach tube. This dietary regime was much the most successful of several tried. On it, the biotin-deficient group showed appreciable deficiency after 3–4 weeks, and thereafter tryptophan (1 mg./g. body wt./day) was added at intervals to the diet of all groups, and the pattern of urinary metabolites studied. At no stage during a period of 10 weeks, in which severe, and in some cases fatal, deficiency became established, was there any appreciable difference between the metabolite excretory pattern of biotin-deficient and control rats. Nor was there any apparent inhibition of the pyridoxin-deficient excretory pattern in the animals with a double deficiency, showing that formation of kynurenine and hydroxykynurenine was not inhibited.

It is therefore considered improbable that biotin has a direct effect on any stage in the conversion of tryptophan into hydroxykynurenine by the rat, though there is a possibility, considered small, that biotin forms part of a coenzyme not readily dissociated from its enzyme in biotin-deficiency.

I thank Mr A. Asatoor for skilled assistance.

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The Metabolism of 3:4-Dichloronitrobenzene in the Rabbit with Special Reference to the Formation of Mercapturic Acids. By H. G. Bray, Sybil P. James and W. V. Thorpe. (*Physiology Department, Medical School, University of Birmingham*)

In a previous communication (Betts, James & Thorpe, 1953) it was reported that mercapturic acids were formed in the rabbit from penta- and 2:3:4:6-tetra-chloronitrobenzene and that this occurred, as with 2:3:5:6-tetrachloronitrobenzene (Bray, Hybs, James & Thorpe, 1953), with the concomitant elimination of the nitro groups. Other chloronitrobenzenes have now been examined with special reference to the possibility of mercapturic acid formation and 3:4-dichloronitrobenzene has yielded two metabolites of special interest.

The major metabolite of this compound is a mercapturic acid which is excreted in an amount corresponding to about 40% of the dose of 3:4-dichloronitrobenzene. This mercapturic acid is, however, formed by elimination not of the nitro group but of the relatively labile (Berckmans & Holleman, 1925) chlorine atom in position 4. This is confirmed by the observation that the same *N*-acetyl-*S*-(monochloronitrophenyl)-L-cysteine is formed from both 3:4-dichloronitrobenzene and 4-bromo-3-chloronitrobenzene. 2:4- and 2:5-Di-

chloronitrobenzenes have provided two further examples of the formation of a mercapturic acid with elimination of a chlorine atom. In both these compounds the lability of the chlorine atom in position 2 (Berckmans & Holleman, 1925) has been confirmed by the isolation of the same *N*-acetyl-*S*-(monochloronitrophenyl)cysteine from 2:4-dichloro- and 2-bromo-4-chloronitrobenzene and of the same *N*-acetyl-*S*-(monochloronitrophenyl)cysteine from 2:5-dichloro- and 2-bromo-5-chloronitrobenzene.

An unusual type of metabolite isolated in small yield (1% of dose) from urine of rabbits given 3:4-dichloronitrobenzene is 3:4:3′:4′-tetrachloroazoxybenzene. Other metabolites were 3:4-dichloroaniline (23% of dose) and a phenol (or phenols) conjugated with glucuronic acid (12%) and sulphuric acid (12%).

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The Analysis of 2-Ethoxycarbonylthio-1-methylglyoxaline. By C. E. Searle. (*Royal Free Hospital School of Medicine, London, W.C.* 1)

McAllister's convenient method for the colorimetric determination of antithyroid drugs of the 2-thiouracil and 2-mercaptoglyoxaline series (1951; McAllister & Howells, 1952) by means of 2:6-dichloroquinone chloroimide is not directly applicable to 2-ethoxycarbonylthio-1-methylglyoxaline ('carbimazole') owing to the absence of a replaceable hydrogen atom until the ethoxycarbonyl group has been removed by hydrolysis. Under the usual experimental conditions (in borate buffer at pH 8·0) carbimazole hydrolyses slowly, but darkening of the reagent occurs before colour development, due to the liberated 2-mercapto-1-methylglyoxaline ('methimazole'), is complete.

McAllister (1955) has recently described a method for analysing carbimazole in an aqueous solution, hydrolysis to methimazole being first carried out in 0·25N-NaOH at 37°. We found that such a method could not be used for estimating carbimazole in tablets as considerable losses of carbimazole occurred. This is attributed to the reducing action in alkaline solution of the lactose present as excipient. Similar losses occurred in Na_2CO_3 solution and when the lactose was replaced by other reducing agents such as glucose, formaldehyde or acetaldehyde, but, as expected, not with sucrose or acetone. Good recoveries were only obtained when the hydrolysis was carried out under very mild alkaline conditions (e.g. in cold 0·05N-NH_4OH).

Acid hydrolysis of carbimazole, which proceeds much less readily than does alkaline hydrolysis, resulted in decomposition of the lactose and loss of the drug.

Carbimazole may also be determined after separation from lactose by extraction from an aqueous solution with chloroform. The more water-soluble methimazole is only partially extracted under these conditions, and the presence of some of this compound in tablets of carbimazole was demonstrated by the action of dichloroquinone chloroimide on the aqueous fraction after extraction.

Some such method of estimating, at least approximately, the relative amounts of methimazole and carbimazole in tissues might help to settle the question of whether carbimazole owes its antithyroid activity to methimazole liberated by *in vivo* hydrolysis, as suggested by Lawson, Rimington & Searle (1951) or whether it is active *per se*, as is indicated by the enzyme inhibition experiments of Fraser, Garrod, Hanno & Jadresic (1954).

I am greatly indebted to Dr A. Lawson for encouragement and advice, and to the Council of the Royal Free Hospital School of Medicine for a Research Fellowship. Some of the work was carried out during the tenure of an I.C.I. Research Fellowship.

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The Biosynthesis of ^{14}C-Labelled Ergocalciferol. By E. KODICEK. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Havinga & Bots (1954) reported the synthesis of [3-^{14}C]cholecalciferol, but the preparation of labelled ergocalciferol has so far not been recorded. Experiments are described in which ^{14}C-labelled ergosterol has been prepared biosynthetically from sterol-deficient yeast (Klein, Eaton & Murphy, 1954; Klein, 1954), which was then incubated with radioactive acetate. The labelled ergosterol was converted by ultraviolet irradiation to ergocalciferol, which, because of its multiple labelling, allows the detection of breakdown products better than cholecalciferol, labelled only in carbon 3.

Saccharomyces cerevisiae, strain LK2G12, obtained through the courtesy of Dr H. P. Klein, was grown in N_2 for 24 hr. at 32°. The harvested yeast cells, having 0·72 mg. ergosterol/g. dry wt., were vigorously aerated for 24 hr. at 32° in 0·1 M-phosphate-acetate buffer, pH 5·2, to which 200 μC of [*carboxy*-^{14}C]acetate had been added. The unsaponifiable matter of the yeast had 1·4% of the total radioactivity, and contained 3·5 mg. [^{14}C]ergosterol/g. dry yeast. The organic acid fraction, yeast residuum, respired CO_2 and medium had, in μC: 14·7, 27·1, 105·4 and 33·3, respectively, representing 84·4% of total radioactivity.

The impure [^{14}C]ergosterol from two experiments, isolated from its digitonide, with a specific activity of 0·08 μC/mg., was diluted 6·8 times with carrier ergosterol and irradiated in dry, peroxide-free ethyl ether with ultraviolet light to 40% conversion. The irradiated material, freed from ergosterol by freezing and digitonin precipitation, was treated with maleic anhydride to remove tachysterol. After partition chromatography on a column of MgO–kieselguhr (Kodicek & Ashby, 1954), the sterols were treated with 3:5-dinitrobenzoylchloride at 24° and the ^{14}C-labelled ergocalciferol ester, of about 97% purity, was isolated by fractional crystallization, after addition of carrier ergocalciferol 3:5-dinitrobenzoate. Total yield of the 3:5-dinitrobenzoate was 14·1 mg., m.p. 141°, $E^{1\%}_{1\,cm.}$ at 265 mμ. in ethanol = 440 after saponification, specific activity 3·4 μC/mM. Small amounts of radioactive lumisterol and suprasterol II were also prepared.

I wish to thank Dr H. P. Klein, University of Washington, for valuable information and Mr D. R. Ashby for his efficient technical assistance.

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The Functional Groupings of the Arylsulphatase of *Alcaligenes metalcaligenes*. By K. S. DODGSON, B. SPENCER and K. WILLIAMS. (*Department of Physiology, University of Wales, Newport Road, Cardiff*)

During the purification of the arylsulphatase of *Alcaligenes metalcaligenes* (see Dodgson, Melville, Spencer & Williams, 1954) a constant arylsulphatase:RNA-phosphorus ratio suggested that RNA was associated with enzyme activity. Furthermore, paper electrophoresis showed that the nucleic acid and arylsulphatase activity possessed similar mobilities. Binkley (1952, 1954) has indicated that the peptidase activities of a cell are a property of the polynucleotides of the cell and it was possible that this might also apply to the arylsulphatase of *Alcaligenes*. However, prolonged electrophoresis resulted in some separation of the nucleic acid and the arylsulphatase and it was possible to elute arylsulphatase, free from nucleic acid, from the paper. The electrophoretic procedure removed much inert protein and other esterases which hydrolysed phenyl acetate and *p*-nitrophenyl phosphate. One g. of the material purified in this way liberated 1 500 000 μg. *p*-nitrophenol from potassium *p*-nitrophenyl sulphate (NPS) in 0·1 M phosphate, pH 8·75, in 1 hr. at 37·5°. The ratio of activities towards NPS, *p*-acetylphenyl sulphate (APS) and nitrocatechol sulphate (NCS) under the respective optimum conditions was 5·0:5·2:1.

When pK_m values (i.e. $-\log_{10} K_m$) were plotted against pH, the three substrates gave almost identical discontinuous curves similar to the types described by Dixon (1953). Interpretation of these curves according to the rules of Dixon indicated the presence of dissociating enzyme-substrate binding groupings with pK's in the region of pH 8·2 and 9·4 respectively. The pK of the free phenolic grouping of NCS in the region of pH 6·5 was reflected in the pK_m/pH curve for this substrate. Groupings present in proteins with pK's in the region of pH 9·4 include ϵ- and α-amino, phenolic and sulphydryl groups. Although 5×10^{-3} M *p*-chloromercuribenzoic acid inhibited the enzyme to the extent of about 50%, sulphydryl groups appear to be excluded from consideration since this

inhibition could not be reversed by cysteine or glutathione. Other evidence indicated this active centre to be ϵ- or α-amino rather than phenolic groups. The nature of the enzyme-substrate binding group with a pK at pH 8·2 is at present obscure, although it may be deduced from pK_m/pH curves that it is a grouping which gains a positive charge (or loses a negative charge) below that pH.

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A Cerebrospinal Fluid Protein Fraction which is not Detectable in Serum. By Elizabeth M. Press. (*S.A. Courtauld Institute of Biochemistry, London*)

The colloidal gold test of Lange gives a high proportion of abnormal (positive) results with cerebrospinal fluid (c.s.f.) from patients with multiple sclerosis (Freedman & Merritt, 1949). The mechanism of the test was studied by Maclagan & Bunn (1947) using albumin and globulin fractions from serum. They demonstrated that γ-globulin coagulated the colloidal gold and albumin, α- and β-globulins protected it. Kabat, Freedman, Murray & Knaub (1950), showed that the concentration of γ-globulin in c.s.f. was increased in patients with multiple sclerosis.

In the present study strong correlation has been shown between the percentage of γ-globulin in the c.s.f. and the colloidal gold test. However, in some cases a very moderate increase in γ-globulin was associated with a strongly positive gold reaction, also serum diluted to 0·03% protein gave a positive gold reaction. This was suggestive of a qualitative as well as a quantitative difference in the protein fractions. The albumin and globulin fractions were therefore isolated from serum and from normal and pathological c.s.f.'s by continuous paper electrophoresis (Hobart & Rose, 1955) and the effect of these fractions on the colloidal gold reaction was studied. γ-Globulin from serum was a stronger coagulant than that derived from normal c.s.f. γ-Globulin from c.s.f. having a positive colloidal gold reaction, was of intermediate coagulating power. Albumin from c.s.f. and serum inhibited coagulation but was only one-tenth as effective as β-globulin. α-Globulin from serum had the same protective power as β-globulin, but α-globulin from all c.s.f.'s was about twice as effective.

Sedimentation constants of the protein fractions were also determined. c.s.f. γ-globulin was found to contain a slowly sedimenting component not detected in serum γ-globulin. The proportion of this component was greater in γ-globulin derived from normal c.s.f.'s. The slowly sedimenting component was found to inhibit the colloidal gold reaction.

γ-Globulin has also been fractionated by 18% sodium sulphate in phosphate buffer, which precipitated 95% of the serum γ-globulin but only 75–85% of c.s.f. γ-globulin. The supernatant protected the colloidal gold and the precipitate coagulated it.

c.s.f. β-globulin also had a slowly sedimenting component not found in serum β-globulin.

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The Effect of Calcium-Depletion on the Chemical Composition of Bone Minerals. By T. G. Taylor and J. H. Moore. (*Department of Agricultural Chemistry, The University, Reading*)

Progressive mineral depletion was induced in pullets by allowing them to lay 2, 4 or 6 eggs on a calcium-deficient diet, and the degree of depletion suffered by individual bones was assessed on their total ash content, compared with corresponding bones from control birds which did not receive the deficient diet (Taylor & Moore, 1954). One group of seriously depleted bones was analysed from each of the 8 experimental birds. Calcium, magnesium, sodium, potassium, phosphate, carbonate, citrate and total ash were determined in the dry, fat-extracted bone powder after it had been freed from non-osseous organic matter by centrifuging with CCl_4.

There was no correlation between the ash percentage of the bones and the extent of mineral depletion, which lends support to the view that when minerals are lost from the skeleton the organic matrix is destroyed simultaneously (McLean & Bloom, 1940).

Mineral depletion resulted in progressive increases in the magnesium and phosphate content of the ash. Marked decreases in the carbonate content and slight decreases in citrate occurred during the periods when active bone resorption was taking place. Very large real increases in the sodium and potassium content of the bone-ash occurred during the calcification of the third and fourth eggs. The potassium content of the bones of the 4-egg birds was almost twice as great as in the 2-egg birds.

In spite of the large differences in the composition of the 8 bone samples, residual Ca:residual P weight ratios varied only from 1·94 to 2·02, the lower values occurring with the most seriously depleted bones.

It is suggested that the observed bone changes were reflections of changes in the serum of the experimental birds, which resulted in changes in the ions adsorbed on to the crystals. The most important blood changes are likely to have been (1) a reduction in the alkali reserve (Common, 1941), (2) a reduction in the ionic calcium (Deobald, Christiansen, Hart & Halpin, 1938), which together caused decreased adsorption of calcium, carbonate and citrate and increased adsorption of phosphate, sodium and potassium.

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Cytochromes from *Chlorobium thiosulphatophilum*. By JANE GIBSON and H. LARSEN (introduced by S. R. ELSDEN). (*A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield*)

The isolation of a cytochrome pigment from *Rhodospirillum rubrum* has been described by Elsden, Kamen & Vernon (1953), and Kamen & Vernon (1954) have also briefly reported the detection of a cytochrome in *Chlorobium limicola*, a strict anaerobe. An investigation of two cytochrome pigments from *Chl. thiosulphatophilum* has now been carried out.

Cytochrome-containing extracts could be made from fresh cells by freezing and thawing, or by treatment with cetyltrimethylammonium bromide. The pigments from dried cells could be extracted with phosphate or borate buffers, pH 6·5–9·2. On reduction with $Na_2S_2O_4$, absorption bands at 554 and 523 mμ. were observed.

The pigments extracted from 150 g. dried cells were separated using Amberlite IRC–50, after Margoliash (1953). A basic component, provisionally named Chlorobium cytochrome 554 (1) remained adsorbed, while the second pigment, Chlorobium cytochrome 554 (2) passed through. This component was purified by treatment with *n*-butanol and by adsorption on cellulose–calcium phosphate gel columns (Elsden, personal communication).

The properties of Chlorobium cytochrome 554 (1) after several treatments with Amberlite, are: 1. The pigment is slowly autoxidizable. 2. In the reduced form, absorption maxima occur at 554, 523 and 417 mμ. When viewed in the hand spectroscope, the edges of the α-band are seen to be sharper than those of animal cytochrome *c*, and the β-band relatively stronger. 3. No CO compound is formed. 4. The oxidized pigment forms an NO compound with absorption bands centred at 573 and 537 mμ. 5. The Fe and haematin content of a sample was 0·37 and 3·1 % respectively. 6. The oxidation-reduction potential lies between the ranges covered by the ferro–ferricyanide and ferro–ferrioxalate systems. A preliminary measurement using 2:6-dichlorophenolindophenol gave $E'_0 \simeq 160$ mv. at pH 7.

The distinction between the two cytochromes rests primarily on the difference in basicity of the proteins, although minor differences in absorption spectrum are also apparent. In other properties the two are similar. Enough pure Chlorobium cytochrome 554 (2) for Fe and haematin estimations has not yet been obtained.

The cytochrome content of the extracts accounts for 0·1–0·3 % of the dry weight of the cells, the two pigments being present in approximately equal amounts. While an exhaustive search for other cytochrome components has not yet been carried out, no other absorption bands have been seen.

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Vitamin B_{12} in Sewage. By J. M. Whitmarsh, J. W. Albans and R. D. Wright. (*Boots Pure Drug Co. Ltd., Nottingham*)

Vitamin B_{12} was shown by Hoover, Jasewicz & Porges (1951) and Hoover, Jasewicz, Pepinsky & Porges (1952) to occur in activated sludge to the extent of 10 μg./g. (dry basis). Later the City of Milwaukee procured a patent (1954) and is reported to have erected a pilot plant.

The figure of Hoover *et al.* (1952) was confirmed for activated, primary and methane digester sludges. The last named is preferred since it is relatively concentrated, 4% solids against 1–2% for the other sludges.

Assayable activity can be extracted by boiling, without the need for autoclaving recommended by Hoover *et al.*, and is destroyed by heating at pH 11. Extracts were concentrated and purified by well-known methods, then chromatographed on alumina or silica columns. Only one pink band was observed. At all stages assays by *Escherichia coli* mutant and *L. leichmannii* had a ratio of unity. It was concluded that activity was probably due to cyanocobalamin although no ionophoresis (Holdsworth, 1953) was attempted. This was before the discovery of B_{12} III by Friedrich & Bernhauer (1953).

Extracts of 40 gal. of sludge were prepared, the spent sludge being removed centrifugally. Preliminary centrifugal concentration of the sludge is inadvisable since at 10% solids the suspension becomes gelatinous and retains too much water. The vitamin in the extracts could be adsorbed on to carbon or other adsorbants by stirring and filtering; in this form it could be used as a feeding stuff supplement, rather than as a starting point for purification to the crystalline vitamin.

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The Electrophoretic Analysis of Hemolysates of Red Cells of Individuals with Sickle Cell Trait. By E. M. Shooter and E. R. Skinner. (*Department of Biochemistry, University College, London*)

Since Pauling, Itano, Singer & Wells (1949) discovered that sickle cell and normal human carbonmonoxyhaemoglobins had different electrophoretic mobilities, electrophoresis has been widely used in the study of abnormal haemoglobins. However, the choice of conventional buffers which suppress boundary anomalies only permits adequate resolution of sickle cell and normal haemoglobins either after long times of migration or alternatively with relatively diffuse boundaries (Pauling *et al.* 1949; Morrison, Rudincki & Diggs, 1954). Hoch (1950) has already noted the advantages of steady state migration for the separation of proteins of similar mobility and has demonstrated this effect in unbuffered systems with adult and foetal haemoglobins.

We have now found that excellent separations of sickle cell and normal haemoglobins are obtained in buffered systems by emphasizing the conductivity and pH effects in order to sharpen the boundaries in one limb. Thus in sodium cacodylate buffer ($I = 0{\cdot}04$, pH = 6·40, protein concentration $\sim 2\%$) the faster ascending boundary migrated in an approximately steady state and complete resolution was obtained in 6 hr. at 7·4 v./cm. Similar results were found with phosphate, phosphate–chloride and ethylenediamine–HCl buffers when the comparisons were made at constant ionic strength and haemoglobin mobility. The variation of mobility at constant pH showed that the affinity of haemoglobin for the buffer anions decreases in the order phosphate : chloride : cacodylate with corresponding changes in isoelectric points.

The same resolution was found in the pH 6·4 phosphate buffer between $I = 0{\cdot}04$ and 0·08 or in the $I = 0{\cdot}04$ buffer from pH 6·4 to 6·9. Under conditions of excessive boundary sharpening at pH 6·0 and $I = 0{\cdot}04$ an additional component was detected whose mobility was slightly higher than that of sickle cell haemoglobin. The boundaries in uncharged acid type (e.g. sodium barbiturate) or phosphate buffers on the alkaline side of the isoelectric points where conductivity and pH effects are in opposition were more diffuse and the resolution therefore inferior. On the other hand in the uncharged base buffer aminotrishydroxymethylmethane–HCl ascending boundaries were again sharpened but became asymmetric after migrating short distances.

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Occurrence of Electrophoretically Distinct Haemoglobins in Ruminants. By H. HARRIS and F. L. WARREN. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

In the course of an investigation of the electrophoretic behaviour of adult and foetal haemoglobins of the goat it was found that certain animals showed the presence of two adult haemoglobins of distinctly different mobilities each of which was easily separated from foetal haemoglobin. The experiments were carried out in filter paper with barbitone buffer, pH 8·6, $I = 0{\cdot}05$, in an apparatus similar to that described by Flynn & de Mayo (1951) and using a potential difference of 185v for 17 hr.

In 26 unselected goats obtained from several different dealers, 10 showed the presence of two haemoglobins, both migrating towards the anode. In the remaining 16 animals only one haemoglobin was observed and this corresponded in mobility to the faster moving haemoglobin in the animals showing two. In 15 samples of blood obtained from foetuses ranging in age from 50 to 120 days only one haemoglobin was found which moved faster than either of the adult haemoglobins. The foetal haemoglobin appeared to be the same irrespective of whether the maternal blood contained one or two haemoglobins.

Bloods from 46 goats of a pedigree herd were also examined. In these animals 8 showed the presence of two haemoglobins while 38 contained only the faster moving haemoglobin.

Of the total of 76 goats so far examined, 66 were females and most of these were pregnant. However, in the non-pregnant goats, animals with one or with two haemoglobins have been found. So far only 6 male goats have been examined and in each of these animals only one haemoglobin has been found.

It has also been possible to examine bloods from 12 pregnant sheep. In this small series 3 types of individuals could be identified. Five animals contained two haemoglobins; three showed a single relatively fast moving haemoglobin; four showed a single relatively slow moving haemoglobin. Eight bloods from foetuses from these ewes were each found to contain one haemoglobin which had a mobility intermediate between the fast and the slow adult haemoglobins.

The possibility that the individual variations in the blood pigments of these ruminants are genetically determined is being further investigated.

We should like to express our thanks to Dr S. J. Folley, F.R.S., and Dr A. T. Cowie for bloods from the pedigree goat herd at the National Institute for Research in Dairying, and to Professor A. St G. Huggett and Dr E. C. Long of St Mary's Hospital Medical School for blood from pregnant sheep.

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The Role of Ornithine and Citrulline in Urea Synthesis. By J. R. BRONK and R. B. FISHER. (*Department of Biochemistry, University of Oxford*)

Gornall & Hunter (1943) found that at low concentrations ornithine was a more effective catalyst of urea synthesis in rat-liver slices than citrulline, which is not to be expected if the Krebs & Henseleit (1932) ornithine cycle is true for this system. Krebs (1952) has suggested that this may be due to difficulty experienced by citrulline in gaining access to the cells.

We have confirmed the observations of Gornall & Hunter and have shown that citrulline penetrates liver water at least as readily as ornithine. Since Cohen & Hayano (1948) have shown that the enzyme system converting citrulline to arginine is in the general cytoplasm, and that the only part played by mitochondria in this process is due to their oxidizing enzyme systems, it is not possible to suppose that the lesser effectiveness of citrulline is due to its exclusion from a fraction of the intracellular water.

We have also shown that even in favourable conditions the catalytic effects of ornithine and citrulline on urea synthesis are not additive; when the two are added together to liver slices the rate of urea synthesis observed is never in our experience greater than the higher rate obtained with one of the two at the same concentration.

On these grounds and on the basis of the differences in response of the ornithine- and citrulline-catalysed processes to various alterations in conditions, we suggest that these processes involve separate cycles.

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The Interaction of Silicic Acid and Collagen and Gelatin Monolayers. By P. F. Holt and S. G. Clark. (*The University, Reading*)

Multipoint interaction of proteins and polyhydroxy compounds has been observed. Ellis & Pankhurst (1954) studied the effect of mimosa tannin on collagen hide-powder monolayers. They observed chelation of the protein by the tannin. Silicic acid has been shown to react with monolayers of gliadin (Schulman & Rideal, 1937), albumin and methaemoglobin (Holt & Bowcott, 1954).

The area occupied by a monolayer may be either increased or decreased by silicic acid in the substrates if the protein and the silicic acid interact. The effect obtained depends upon the conditions under which the protein is spread. When the protein is spread from an efficient spreading solvent, on to a large surface area, spreading is rapid and interaction does not take place until the monolayer has formed. If the protein is spread from a less efficient spreading solvent on to a relatively small surface area the silicic acid chelates the protein and prevents it from uncoiling, thus decreasing the specific area.

This effect has been used to study the interaction of silicic acid and gelatin, hide powder and tendon collagens. Force and area characteristics of the monolayers were followed over a range of pH values. Gelatin, hide powder and tendon collagen films were very compressible and all gave similar force/area curves. When substrates were free from silicic acid little variation with pH of the specific areas was observed and this was also true if the substrate contained unpolymerized silicic acid. On substrates containing polymerized silicic acid, the areas occupied by the gelatin and hide powder collagen films were greatest when the pH of the substrate was about 6, but films of tendon collagen showed maximum specific areas at pH 7–7·5, and an additional peak occurred in the area-pH curve at pH 5·5.

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DEMONSTRATION

A Case of Cryoglobulinaemia. By E. H. Hemsted, E. W. Ingarfill and A. L. Tárnoky. (*Royal Berkshire Hospital, Reading*)

An obscure normocytic normochromic anaemia was found in a woman aged 82. Sternal marrow biopsy showed 16% atypical plasma cells. Proteinuria was slight; Bence-Jones proteose was absent. The serum contained a protein fraction which solidified on cooling to 4°, and dissolved again with no apparent change when warmed to room temperature.

The following results were obtained with total and residual serum (separated from the abnormal fraction at 0°):

Total serum: Immediate direct Van den Bergh, negative; bilirubin 0·1 mg./100 ml. Phosphatase (King-Armstrong), alkaline 8, acid 0·5. Calcium ($KMnO_4$ titre) 18·8, urea 40, inorganic P 4·5, cholesterol (total) 175, (free) 45, (mg./100 ml.). Turbidities: ethanol (Love & Mawson, 1948) 0·2, thymol 0·3, zinc sulphate (Maclagan, 1951) 7·9. Thymol flocculation was negative. Protein content (Robinson & Hogden, 1940) 7·6 g./100 ml. Albumin 2·2 and globulin 5·4 g./100 ml.

Residual serum: Calcium 9·2 mg./100 ml. Cholesterol (total) 225 and (free) 65 mg./100 ml.

The serum gave weak euglobulin and formol gel tests. On filter-paper electrophoresis (Flynn & de Mayo, 1951) it showed a strong fibrinogen-like band between the β- and γ-globulin positions. This was still present, though much weaker, in residual serum and in urine dialysed against dextran. Two-dimensional electrophoresis of the serum failed to resolve the abnormal band any further; in 1M-NaCl it showed two components, one precipitating at pH ~ 5·8, the other at pH ~ 4·4. Cold fractionation (MacKay & Kekwick, 1954) isolated a pure component resembling β-globulin in mobility. Neither fat nor carbohydrate could be demonstrated in this material.

Quick's one-stage prothrombin estimation could not be carried out since the blood clotted when taken into citrate. Using twice the prescribed amount of citrate the prothrombin time was 15 sec. (normal, 11 sec.). The abnormal fraction did not behave like fibrinogen when thrombin was added to oxalated plasma, serum, or serum enriched in cryoglobulin.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **341st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Oxford, on* 18 *June* 1955, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

A Transitory, Rapid, Production of Lactate in Electrically Excited Cerebral Tissues. By H. McIlwain. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

Cerebral tissues in 2·5 or 10-mM-glucose salines form in an hour about 20 μmoles of lactate/g., increased by electrical pulses to about 100 μmoles/g. However, by applying pulses for a few seconds only, a transitory formation at the rate of 420 μmoles/g./hr. has been observed. This lasts for only 20 or 30 sec., and the tissue content of lactate, which has risen from 1·5 to 4 μmoles/g., then falls. If pulses are stopped within the first 20 sec. while lactate is still increasing, the increase continues for some seconds after pulses have ceased. During the rise in lactate, the tissue's glycogen remains steady but glucose falls by an equivalent amount. However, supply of glucose does not limit the lactate formation, for the tissue itself, respiring in 10 mM glucose, contains 7·3 μmoles of glucose/g.

In interpreting both the slowing of glycolysis and its 'inertia', it is to be noted that the initial rate of glycolysis approaches that of a brief production of inorganic phosphate from labile phosphates (Heald, 1954). This change lasts only 5–7 sec. and leaves labile phosphates still in the tissue. Thus its cessation after 5 sec. could be due largely to the phosphorylation associated with glycolysis (at 1 mole P/mole lactate); but it does not recommence when glycolysis decreases. Presumably respiration has by then increased; it is known to do so within 1 or 2 min. of commencing pulses, by 60–70 μmoles O_2/g./hr., again probably esterifying about 400 μmoles phosphate/g./hr.

Most experiments have been carried out with guinea-pig cerebral cortex, but that from the rabbit, rat and man has been observed to behave similarly in several respects.

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The Effect of Electrical Pulses on the Distribution of Radioactive Phosphorus in Cerebral Slices. By P. J. Heald. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

Previous studies (Heald, 1954) have shown that the rate of breakdown of creatine phosphate in slices of cerebral tissue, brought about by electrical pulses, would release 'energy-rich' phosphate at a rate far greater than could be accounted for by the rate of phosphorylation normally occurring in the metabolism of glucose. It was suggested that possibly some other phosphorylation process was occurring. Attempts to follow the fate of creatine phosphate during breakdown have been carried out using radioactive phosphorus. Slices of guinea-pig cerebral cortex held in plastic grid electrodes (Heald, McIlwain & Nelligan, 1954) were incubated in an oxygenated aminotrishydroxymethylmethane-glucose-saline for 30 min. Carrier-free radioactive phosphate was added, and after incubation for 3 min. the tissues were removed, briefly washed in warm oxygenated saline and re-incubated in fresh saline. After a period of time, determined as suitable by preliminary experiments, condenser pulses (Ayres & McIlwain, 1953) were passed for 10 sec. The slices were immediately fixed in trichloroacetic acid and the acid-soluble phosphates were fractionated by a method based on paper ionophoresis (Heald, 1955). In tissues exposed to pulses the relative specific activity (counts/min./μg. P relative to incubation medium specific activity (counts/min./μg. P) of 2×10^4) of inorganic phosphate fell from an average value of 199·3 to 160·0, that of the γ-P of adenosine

triphosphate from 110·6 to 43·6 and that of creatine phosphate from 92·0 to 40·0. Accompanying the decrease the relative specific activity of the acid insoluble phosphates increased. The results suggest that a rapid exchange takes place, possibly between adenosine triphosphate and a tissue component, accelerated during the passage of electrical pulses.

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Fatty Acid Synthesis in Soluble Enzyme Preparations. By PRISCILLA HELE and G. POPJÁK. (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

A soluble enzyme system from rat mammary gland, which synthesizes fatty acids from labelled acetate, has previously been described (Popják & Tietz, 1953, 1954, 1955; Tietz & Popjak, 1955). The synthesis of fatty acids in this system requires adenosine triphosphate (ATP) and coenzyme A (CoA), and is greatly enhanced by the concomitant oxidation of α-oxoglutarate.

A similar system has now been obtained from rabbit mammary gland. The extract is prepared as described previously (Popják & Tietz, 1953, 1955), then the protein precipitated by 90% saturation with ammonium sulphate. The activity of this preparation/mg. protein can be increased some 4- to 5-fold by refractionation between the limits of 35–60% saturation with ammonium sulphate.

It has been possible, in rabbit mammary gland preparations, to replace completely α-oxoglutarate as electron donor in the synthesis of fatty acids with reduced diphosphopyridine nucleotide (DPNH). No significant synthesis of fatty acids occurs in the absence of DPNH.

Fatty acid synthesis from DPNH and acetate can be followed spectrophotometrically by observing the decrease of optical density at 340 mμ. Reduced cytochrome *c* + DPN cannot replace DPNH as electron donor in this system. A typical assay system contains 0·4 μM DPNH, 2 μM CoA, 30 μM cysteine, 30 μM ATP, 20 μM $MgCl_2$, 100 μM Tris buffer pH 9·4, 10–60 μM acetate, and 10 mg. protein, in a total volume of 3 ml., final pH 8·0. The reaction mixture, less acetate, is incubated at 38° for 5 min. to eliminate small blank reactions utilizing DPNH. The reaction is started by the addition of acetate. No significant disappearance of DPNH occurs in the absence of ATP, CoA, acetate or cysteine, the rate of the reaction being dependent upon their concentrations and upon the concentrations of DPNH and of protein. In 40 min. the system outlined above will utilize 0·04 μM acetate and 0·2 μM DPNH in the synthesis of fatty acids.

During the early part of the reaction, which is linear with time, the ratio DPNH:acetate utilized for fatty acid synthesis is about 6:1, but this falls as the reaction proceeds. When half the DPNH has been utilized synthesis stops, irrespective of the starting concentration of DPNH, or of enzyme concentration, but can be re-started 'explosively' when further DPNH is added. This is associated with a fall in the DPNH:acetate ratio towards 2, suggesting the complete utilization of intermediates formed during the preceding phase. The reaction can be reversed by adding high concentrations of DPN.

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A Manometric Procedure for the Study of Enzymic Transmethylation Reactions involving Thetins. By G. A. MAW. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Transfer of methyl groups from betaine and dimethylthetin to homocysteine has been shown to take place in tissue slices and suspensions (Borsook & Dubnoff, 1947; Dubnoff & Borsook, 1948). In these experiments the methionine formed was estimated colorimetrically. Consideration of the overall reactions involved shows that in each case methylation of homocysteine is accompanied by the liberation of a proton. With the thetin as the methyl donor, if the reaction is carried out in a bicarbonate buffer, it is possible to follow it manometrically by the rate of CO_2 liberation. Experiments with rat liver suspensions at pH 7·4 have shown that the volume of CO_2 formed is equivalent

to the amount of methionine synthesized. The procedure resembles that used by Smythe (1936) for following the non-enzymic reaction between iodoacetate and various thiols in a bicarbonate buffer.

With betaine as the methyl donor, methionine synthesis is accompanied by the formation of equivalent amounts of the base dimethylglycine, which neutralizes the acid formed in the reaction. Methionine synthesis from homocysteine and betaine in rat liver suspensions is therefore not accompanied by CO_2 production.

The manometric procedure has been used in a study of the thetin-homocysteine transmethylase of rat liver. The enzyme has been concentrated by ammonium sulphate fractionation and precipitation with ethanol at 0°. At pH 7·4, which is close to the pH optimum of the enzyme, as determined by Dubnoff & Borsook, the optimum concentration of DL-homocysteine was found to be approx. 0·03M. The optimum concentration of dimethylthetin has not been established with certainty, since enzyme activity was not completely maximal with concentrations of the thetin chloride as high as 0·3M, indicating a low affinity of the enzyme for this substrate. The transmethylase is unaffected by the presence of cysteine, but is inhibited by thioglycollate.

'Active methionine' or *S*-adenosylmethionine (Cantoni, 1952; Baddiley & Jamieson, 1954) may also be considered as a thetin. The methylation of glycocyamine by this compound should involve liberation of a proton and in view of the weakly basic nature of the resulting creatine, it should also be possible to follow this reaction manometrically in a bicarbonate medium. The methylations of nicotinamide and dimethylethanolamine do not appear to be reactions amenable to such study since neither involves proton formation.

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Interference by Sulphide with Inorganic Phosphate Determinations by the Method of Lowry & Lopez and its Elimination by the Addition of Copper Ions. By J. L. PEEL, MARJORIE FOX and S. R. ELSDEN. (*Agricultural Research Council Unit for Microbiology and Department of Microbiology, Sheffield University*)

In presence of phosphate, molybdic acid is reduced by ascorbic acid at pH 4 giving a blue colour the intensity of which is proportional to the amount of phosphate present. This principle is the basis of the method described by Lowry & Lopez (1946) for the determination of inorganic phosphate in presence of labile phosphate esters. When this method was applied to mixtures containing extracts of a rumen bacterium, no colour developed, although phosphate was known to be present (Lowry & Lopez had previously observed that colour development was delayed in presence of certain tissue extracts). The bacterial extracts contained Na_2S added during preparation and the effect of sulphide alone was therefore examined. 0·02 μmole Na_2S (final concentration 2×10^{-6}M) completely inhibited colour development in the presence of about 1 μmole of inorganic orthophosphate. This extreme sensitivity to sulphide suggested interference with some catalytic mechanism. The same concentration of Na_2S had no effect on colour development under the conditions of Fiske & Subbarrow (1925) in which the reducing agent is 1-amino-2-naphthol-4-sulphonic acid, suggesting that sulphide inhibition is peculiar to reduction by ascorbic acid.

It was found that the addition of cupric ions (*ca.* 4×10^{-6}M) overcame the inhibitory effect of both Na_2S and of the bacterial extracts. The addition of traces of copper sulphate to the reagents described by Lowry & Lopez is therefore recommended. In presence of higher concentrations of cupric ions (e.g. 2×10^{-4}M), the intensity of the blue colour produced with a given amount of phosphate is substantially increased and this increases the sensitivity of the method.

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The Separation of Substances on the Basis of their Molecular Weights, Using Columns of Starch and Water. By G. H. LATHE and C. R. L. RUTHVEN. (*Research Laboratories, Queen Charlotte's Maternity Hospital, London, W.* 6)

Colloidal carbon, which cannot penetrate starch granules, was used to measure the volume of the mobile phase (4 ml./10 g. starch) on columns of potato starch in water. Uncharged molecules were eluted, after carbon, in order of decreasing size. The R values, relative to carbon, were as follows (mol.wt. in parentheses)—amylose (*ca.* 160000) 0·98, soluble laminarin (*ca.* 3600) 0·96, inulin (*ca.* 5700) 0·93, cyanocobalamin (*ca.* 1300) 0·91, *cyclo*-maltoheptaose (1134) 0·86, *cyclo*-maltohexaose (972) 0·84, raffinose (504) 0·75, sucrose (342) 0·62, glucose (180) 0·57, urea (60) 0·42. Overlapping of the larger substances was marked, especially above mol.wt. 1000 (75 g. column).

Separation of large molecules, charged and uncharged, was greatly facilitated by using maize starch which had been swollen in water at 61–68° for 75 min. until the centrifuged starch volume was 3 times the original. The following R values (0·025 M borate buffer at pH 8·5; 0·025 M KCl) were found—amylopectin (1–400 millions) 0·94, globulin (150000) 0·86, haemoglobin (67000) 0·77, soluble laminarin (*ca.* 3600) 0·64, inulin (*ca.* 5700) 0·58, bacitracin A (*ca.* 1470) 0·48, cyanocobalamin (*ca.* 1300) 0·41, fructose (180) 0·40, urea (60) 0·38.

Lindqvist & Storgårds (1955) reported that starch in citrate at pH 6·7 separated peptides and amino acids in the range of mol.wt. 200–500, and that this was due to a definite 'pore diameter' in the starch granules. A study of individual peptides, amino acids and charged molecules in this range suggests a more complex behaviour, in which the influence of pH and salt tends to obscure the effect of size.

The possibility that the separation of substances in the order of their molecular weights is a true partition effect, due to differing relative solubility in water and hydrated starch (Martin, 1950), was examined by (1) determining the relative solubility in water and 87% (w/v) sucrose solution, of substances of high, and of low R value; and (2) by determining the effect on the R value of using a mobile phase of 87% (w/v) sucrose, instead of water. The results were inconsistent with a partition theory based on solubility. This 'molecular sieve effect' (Synge, 1953) is probably due to the greater depth to which smaller molecules penetrate the starch granules. It can be used to separate materials, and to determine their approximate molecular weight.

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The Utilization of Valine for Penicillin Biosynthesis. By H. R. V. ARNSTEIN and MARGARET E. CLUBB. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Both DL-[γ-^{14}C]valine (Arnstein & Grant, 1954) and DL-[*carboxy*-^{14}C]valine (Stevens, Vohra & DeLong, 1954) are incorporated into the penicillamine moiety of penicillin. The utilization of valine for penicillin has now been further studied by adding valine containing both ^{14}C and ^{15}N to growing submerged cultures of *Penicillium chrysogenum* WIS 48–701 or WIS 51–20 and measuring the incorporation of both isotopes into penicillin and into the valine of the mycelial protein.

The penicillin was isolated and degraded essentially as previously described (Arnstein & Grant, 1954), and in one experiment the distribution of ^{14}C in the penicillamine was determined by desulphurizing the *iso*propylidene derivative and degrading the valine thus obtained to CO_2 and *iso*-butyraldehyde, which was isolated as the 2:4-dinitrophenylhydrazone.

The specific radioactivity of the penicillin was usually about 10% of that of the added valine, and the mycelial valine always had a slightly lower radioactivity than the penicillin. The ^{15}N:^{14}C ratios of the added valine, mycelial valine and penicillamine in a typical experiment were 12·7, 3·0 and 2·4, respectively. There was no striking difference in the incorporation of ^{14}C from labelled L- or DL-valine into penicillin.

Degradation of the penicillamine moiety of penicillin from a fermentation to which uniformly ^{14}C-labelled L-valine had been added showed that the carbon skeleton of valine was used intact, the ratios of radioactivity in the total molecule/carbon atoms 2–5 of the added valine, mycelial valine and penicillin being 1·22, 1·20 and 1·22 respectively. These results are in agreement with the work of Stevens *et al.* (1954), who compared the radio-

activity in the total molecule and in the carboxyl carbon atom of penicillamine and added valine.

It is concluded from the similar utilization of ^{14}C-labelled L- and DL-valine, as well as from the low incorporation of ^{15}N from DL-[^{15}N]valine into penicillin, that D-valine is probably not used directly for penicillin biosynthesis. It is possible, therefore, that dimethylpyruvic acid or a related deamination product of valine may be a more immediate penicillin precursor than is valine.

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Microbial Oxidation of p-Cresol and Protocatechuic Acid. By S. DAGLEY and M. D. PATEL. (*Department of Biochemistry, University of Leeds*)

A strain of *Pseudomonas* sp. able to utilize *p*-cresol as sole source of carbon was isolated from gasworks effluent, and was examined bacteriologically by Dr G. M. Williamson. Cell-free extracts prepared by the Hughes (1951) press attacked *p*-hydroxybenzoic and protocatechuic acids rapidly, and *p*-cresol and *p*-hydroxybenzaldehyde more slowly. Crushes taken up in phosphate buffer were centrifuged at 103000 *g* for 30 min. to give a well-defined layer of pink particles above cell debris: the enzymically active supernatant also contained finer pink particles. In a reversion spectroscope suspensions of whole cells, and of 'coarse' pink particles, showed bands at 555 and 525 mμ. which disappeared on aeration: similar changes occurred in absorption spectra of supernatants plotted from spectrophotometer measurements. Addition of pink particles to extracts increased rates of oxidation of *p*-cresol and *p*-hydroxybenzaldehyde. When the precipitate from ammonium sulphate saturation was taken up in phosphate buffer the solution (soln. A) oxidized protocatechuate rapidly, and *p*-cresol was converted slowly to *p*-hydroxybenzaldehyde; the latter, and *p*-hydroxybenzoate, were not attacked. Oxidation of the aldehyde (and cresol) to *p*-hydroxybenzoate by soln. A proceeded on addition of DPN. Parallels exist between these observations and those of Gunsalus, Gunsalus & Stanier (1953) concerning the oxidation of mandelic acid, and they are also in agreement with a scheme for *p*-cresol oxidation proposed by Smith, Jones & Evans (1952) who worked with whole cells. After dialysis, sol. A was unable to oxidize protocatechuic acid: activity was restored completely by addition of 10^{-3}M Fe^{2+} but not by several other ions investigated. In this respect our preparations differ from those of Stanier & Ingraham (1954) who could report no cofactor for protocatechuic oxidase, but it is of interest that the oxidases of catechol and homogentisic acid require Fe^{2+} (Suda, Hashimoto, Matsuoka & Kamahora, 1951; Suda & Takeda, 1950). Protocatechuic acid was decomposed by the uptake of one O_2/molecule with negligible CO_2 evolution. Since the absorption spectrum of the product did not correspond to that of 'active' β-carboxymuconic acid it might be suggested that the reaction ceased at a point in the sequence between this compound and the decarboxylation reaction.

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Some Quantitative Aspects of the Evaluation of Plasma Proteins following Paper Electrophoresis. By E. M. ABDEL-WAHAB and D. J. R. LAURENCE. (*Postgraduate Medical School of London, Ducane Road, London, W.* 12)

The separation of proteins by electrophoresis on filter paper followed by staining and densitometry is a simple micromethod for obtaining relative protein concentrations and an albumin/globulin (A/G) ratio can be derived directly. By using a densitometer with a linear response (Sweet, 1946) a continuously recorded electrophoretic curve is obtained equivalent in principle to the Philpot-Schlieren photograph.

Simultaneous parallel separations show that the A/G ratio is reproducible (coefficient of variation $<3\%$) and therefore small differences between techniques can be revealed by multiple determinations. The dyes Amidoschwarz 10B, Bayer (Grassmann, Hannig & Knedel, 1951), Lissamine Green, I.C.I., and Bromophenol Blue (Griffiths, 1952) were compared. No significant differences could be found between the A/G ratios with these

dyes or in the albumin/α_2 globulin ratios. The dye uptake and A/G ratios with Amidoschwarz and Lissamine Green are independent of the time of dyeing and dye concentration over at least a ten-fold range.

As Lissamine Green is easily eluted into aqueous buffer, pH 9·2, the results of densitometry can be compared with those of elution. A method of indexing the corresponding segments for elution and densitometry has been developed. No significant difference between elution and densitometry was found and the use of Beer's Law is therefore justified.

No significant differences were found between A/G ratios obtained in phosphate, borate and veronal buffers, although the separation of the α_1 globulin is only complete in the veronal buffer. The most suitable buffers do not have the same ionic strengths. Tests of Azocarmine B were discontinued when it was found that the results were not reproducible, that the dye uptake and A/G ratio were dependent on the dye concentration, and that the dye was split by electrophoresis into two components.

Lissamine Green is recommended for convenience and economy of application but Amidoschwarz 10B gives a more robust method.

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Inhibition of Succinic Oxidase by Antimycin A. By M. B. Thorn. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Antimycin A (Dunshee, Leben, Keitt & Strong, 1949) powerfully inhibits succinic oxidase, without significantly affecting succinic dehydrogenase or cytochrome oxidase (Ahmad, Schneider & Strong, 1950). As shown by the latter workers, the degree of inhibition depends upon the amount of enzyme preparation present, and changes rapidly from slight to complete inhibition with decreasing quantities of enzyme preparation. Potter & Reif (1952), after further study, concluded that antimycin A inhibits a component of the succinic oxidase system, probably the BAL-sensitive factor (Slater, 1949*a*).

In the present work, these characteristics of inhibition of succinic oxidase by antimycin A were confirmed, using heart-muscle preparations (Keilin & Hartree, 1947; Bonner, 1954). When the Q_{O_2} of heart-muscle preparations was decreased by partial inhibition with antimycin A, the degree of inhibition by malonate of the remaining succinic oxidase activity diminished correspondingly, recalling the behaviour of succinic dehydrogenase when the Q_{O_2} is varied by using different methylene blue concentrations (Thorn, 1953). In similar experiments with *p*-aminophenylarsenoxide, which inhibits solely by its action on succinic dehydrogenase (Slater, 1949*b*), inhibition by malonate of the remaining succinic oxidase activity did not diminish. This suggests that the Q_{O_2} alters, in the case of antimycin A, because of changes in the activity of all the enzyme present, and in the case of the arsenical, because of changes in the concentration of fully active enzyme. Antimycin A therefore appears to alter the effective concentration of an intermediate hydrogen (electron) acceptor available to all the 'succinic dehydrogenase' present, whereas the arsenical alters the concentration of the succinic dehydrogenase itself.

In order to investigate the availability of the antimycin-inhibitable factor to the 'succinic dehydrogenase', equal amounts of two preparations of particles, one inhibited 98% by antimycin A, the other 96% by *p*-aminophenylarsenoxide, were mixed. The mixture had considerable succinic oxidase activity, which was accounted for by equal redistribution of the antimycin A throughout the mixture, rather than by interaction between electron-transport systems in differently inhibited particles. It is therefore concluded that the antimycin-inhibitable factor in one enzyme particle is available only to the 'succinic dehydrogenase' in the same particle.

The author is gratefully indebted to Professor F. M. Strong for a gift of antimycin A, and to Dr E. C. Slater for a gift of *p*-aminophenylarsenoxide.

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The Effect of a Fat Meal on the Concentration of Free Fatty Acids in Human Plasma. By D. S. Robinson, P. M. Harris, J. C. F. Poole and G. H. Jeffries. (*Sir William Dunn School of Pathology, University of Oxford*)

The fate of the particulate lipid which enters the blood in man during fat absorption is unknown. In the rat, however, a plasma lipolytic system, active against triglycerides containing long-chain fatty acids, has been demonstrated during the absorption of olive oil. This system has been shown to bring about the dissolution of chylomicrons *in vitro* with the release of free fatty acid (Robinson, Jeffries & French, 1954). In the present study an increase in the free fatty acid content of human plasma during fat absorption has been demonstrated.

Plasma samples obtained from healthy young men, aged 20–30 years, after a 12 hr. fasting period, and 4–5 hr. after the consumption of 250 ml. of cream, were analysed for free fatty acids. An increase in the free fatty acid concentration in the plasma occurred during the absorption of cream. This increase, over the fasting level, varied between 0·4 and 1·4 m-equiv. fatty acid/l. of plasma. A similar increase occurred in the plasma of one subject who drank 200 ml. of olive oil.

When plasma samples obtained after drinking cream were incubated at 37° a further progressive increase in the content of free fatty acids occurred. This varied between 0·25 and 0·47 m-equiv. fatty acid/l. of plasma/hr. incubated.

By steam distillation techniques, it was shown that short-chain fatty acids probably formed only a small part of the total free fatty acids found.

This work was carried out during the tenure of the Alan Johnston, Lawrence and Moseley Research Fellowship of the Royal Society by D. S. Robinson, and of the Locke Research Fellowship of the Royal Society by J. C. F. Poole. A personal grant from the Albert and Mary Lasker Foundation Inc. to P. M. Harris is gratefully acknowledged.

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The Production of Succinate from Propionate by Carbon Dioxide Fixation in Sheep Rumen Epithelial Tissue. By R. J. Pennington and T. M. Sutherland. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

From the observed effects of CO_2 tension on propionate metabolism in sheep rumen epithelial tissue and from other evidence it was suggested that propionate might be metabolized by way of a carbon dioxide addition to give succinate in this tissue (Pennington, 1954; Pennington & Sutherland, 1954).

Propionate was incubated in the presence of malonate with sections of isolated epithelial tissue under an atmosphere of 80 % O_2/20 % CO_2; $^{14}CO_2$ and [1-^{14}C]propionate were used in different experiments. About 85 % of the carboxyl of the propionate metabolized appeared as CO_2. The remainder was found in succinate. CO_2-carbon was incorporated into succinate to the same extent as the carboxyl-C of the propionate. There was little incorporation of CO_2 carbon into succinate in the absence of propionate. Lactate production was small.

These experiments afford strong evidence for the formation of succinate from propionate by a direct pathway involving carbon dioxide fixation. The specific activity of the succinate formed was consistent with this hypothesis. The metabolism of carboxyl-labelled propionate through a pathway involving pyruvate and reactions of the citric acid cycle would result in the formation of unlabelled succinate. The carboxyl carbon would be lost as CO_2.

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The Incorporation of Glycine Carbon into β-Carotene in *Phycomyces blakesleeanus*. By G. Mackinney, C. O. Chichester and T. Nakayama (introduced by T. W. Goodwin). (*Department of Food Technology, University of California; Berkeley and Davis, California, U.S.A.*

Although leucine stimulates β-carotene synthesis in *Phycomyces blakesleeanus*, isotope experiments showed that neither the carboxyl nor the α-carbon atom is preferentially utilized, which means that a decarboxylated 5-C fragment from added leucine cannot be readily incorporated into the carotene skeleton (Chichester, Nakayama, Mackinney & Goodwin, 1955).

[*carboxy*-^{14}C]- and [α-^{14}C]-Glycine have now been tested in a medium containing 8 % (w/v) glucose, inorganic salts and thiamine. The glucose level is high (compared with the usual 2·5 %) because

glycine cultures otherwise grow slowly and little or no effect is observed on carotene production. In each experiment 100 μc contained in 6·5 mg. [*carboxy*-^{14}C]glycine and in 8 mg. [α-^{14}C]glycine were added to 24 plates grown for 10 days and 6–8 mg. of crystalline β-carotene were obtained without the aid of carrier. The specific activities (c.p.m./mg. $BaCO_3$) of medium and β-carotene were: with [*carboxy*-^{14}C]glycine, 440 and 23; and with [α-^{14}C]glycine, 347 and 840. The CO_2 evolved in each experiment was collected and assayed daily for activity beginning with the 3rd day. The c.p.m./mg. $BaCO_3$ were: with [*carboxy*-^{14}C]glycine, 1590, 5340, 4400, 2210, 1110, 690 and 485; and with [α-^{14}C]glycine, 380, 964, 1210, 1280, 740, 596 and 480. It would seem that there is definite enrichment of C_a of glycine in the β-carotene.

Granick (1954) has demonstrated a pathway for the incorporation of δ-aminolaevulic acid (ALA) into porphyrins. Without speculating on a possible connexion between the two syntheses, we may report some experiments on the effect of ALA (kindly supplied by Dr J. R. Dice, The Parke-Davis Co., Detroit, Michigan, U.S.A.) on *P. blakesleeanus*.

We used a low sugar (2·5 %, w/v) medium as control and replaced asparagine with increasing amounts (up to 50 % on a weight basis) of ALA. Growth was slightly decreased only at the highest level of ALA used, whereas β-carotene production increased progressively from 245 μg. (control) to 580 μg. (50 %, ALA). ALA thus exerts an effect at least superficially similar to that of leucine.

Using the same glucose level (2·5 %, w/v) a combination of glycine and succinate yielded the same dry wt. (0·559 g./5 plates) as the asparagine control (0·576 g./5 plates), whereas the glycine-ALA combination gave an appreciably lower value (0·389 g./5 plates). β-Carotene synthesis was not significantly different in these three experiments.

A grant from the National Science Foundation is gratefully acknowledged.

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The Influence of Ethylenediaminetetra-acetic Acid on the Excretion of ^{65}Zn by the Rat.

By Jean M. Millar, M. Isabel Fischer, C. A. Mawson and P. V. Elcoate. (*Biology Branch, Atomic Energy of Canada Ltd., Chalk River, Ontario*)

The prostate glands of man and rabbit, and the dorso-lateral prostate of the rat, contain very high concentrations of zinc (Mawson & Fischer, 1952). Turnover experiments have shown that ^{65}Zn enters the rat dorso-lateral prostate very rapidly and is present in the gland in mobile form. The possibility that radioactive zinc might serve as a source of local irradiation for prostatic cancer is limited by the fact that ^{65}Zn is a γ and e^+ emitter with a half-life of 250 days, which concentrates not only in the prostate but also in bone and in the Paneth cells of the small intestine. Millar, Fischer, Mawson & Elcoate (1954) have shown that ethylenediaminetetra-acetic acid (EDTA) greatly increases excretion of zinc in rat urine, though they did not observe much reduction in the zinc content of individual organs. It was thought possible that if a dose of ^{65}Zn was given, followed later by several injections of EDTA, the most recently deposited zinc might be preferentially excreted. The turnover curves suggested that such an effect would be most likely if the EDTA was given within 2 days of administration of ^{65}Zn.

Two experiments were carried out in which rats received 30 μg. Zn (~30 μc ^{65}Zn) intravenously. In one experiment four daily injections of 150 mg. $CaNa_2EDTA$ were given intraperitoneally, starting 24 hr. after the injection of ^{65}Zn. In the other, the rats received 300 mg. $CaNa_2EDTA$ daily for 4 days, but injections were not commenced until 7 days after the dose of ^{65}Zn. Appropriate controls were used in both cases.

In both experiments the rats receiving EDTA had a much increased urinary excretion of ^{65}Zn, but this was to a great extent compensated by a reduction in faecal excretion. In the experiment in which ^{65}Zn was given 24 hr. before commencement of EDTA the urinary + faecal excretion of ^{65}Zn was 28 % of the administered dose, whereas the controls excreted a total of 23 %. In the experiment where EDTA was started 7 days after ^{65}Zn, total excretion during the 4-day period of EDTA dosage was 10·5 %, compared with 7·5 % for the controls.

It remained possible that the rather small differences in total excretion might have arisen mainly from the organs in which zinc is especially concentrated, but estimation of ^{65}Zn in dorso-lateral prostate, ileum and bone showed no significant reduction in concentration in the animals which had received EDTA.

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The Enzymic Hydrolysis of α-Cocaine. By H. Blaschko and Jean M. Himms. (*Department of Pharmacology, University of Oxford*)

α-Cocaine (methylbenzoyl-α-ecgonine) was first made by Willstätter (1896); it has recently been prepared again in this department (by Foster, Ing and Varagic) for pharmacological study. The enzymic hydrolysis of α-cocaine was studied, as it is known that cocaine is hydrolysed in rabbit serum (Glick & Glaubach, 1941).

α-Cocaine was found to be hydrolysed by rabbit serum, more rapidly than cocaine; however α-cocaine was also hydrolysed by horse serum and by human plasma, preparations which do not act upon cocaine.

The hydrolysis of α-cocaine is sensitive to eserine. With an initial concentration of 0·005 M α-cocaine and 10^{-7} M eserine, the inhibition of horse serum was about 55 %, that of human plasma about 75 %. With horse serum, some of the hydrolysis of α-cocaine appeared to be due to a second enzyme less sensitive to eserine, as even with 10^{-3} M eserine about 20 % of hydrolysis was still observed.

It seems possible that the eserine-sensitive hydrolysis of α-cocaine is catalysed by 'pseudo' cholinesterase: the hydrolysis of α-cocaine in ox serum was very slow, and ox serum is known not to have 'pseudo' cholinesterase activity (Mendel, Mundell & Rudney, 1943).

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Vitamin B_{12} and Methyl Synthesis in *Ochromonas malhamensis*. By B. Connor Johnson,* E. S. Holdsworth, J. E. Ford, J. W. G. Porter and S. K. Kon. (*National Institute for Research in Dairying, University of Reading*)

Work with baby pigs has shown that in vitamin B_{12}-deficient and normal baby pigs vitamin B_{12} takes no part in transmethylation but is required for methyl synthesis from glycine (Johnson, Firth & Mistry, 1955; Chang & Johnson, 1955).

In the hope that the protozoan *Ochromonas malhamensis*, which has an absolute requirement for vitamin B_{12}, might serve as a guide to further work on the function of vitamin B_{12} in higher animals and man, the problem of methyl synthesis and the role in it of vitamin B_{12} was investigated with this organism. With a simple medium containing ammonium chloride, glucose, tween 80, thiamine, biotin and minerals, methionine gave a marked growth stimulation in the presence of vitamin B_{12} and a significant growth response in its absence. This stimulation was not obtained with homocysteine or homocystine. However, in the presence of vitamin B_{12}, homocysteine or homocystine plus glycine or plus serine did replace methionine. In the absence of the vitamin these methionine precursors were inhibitory. In a recent preliminary report of similar studies Miller & Yeager (1955) failed to obtain replacement of methionine by homocysteine plus glycine in the presence of vitamin B_{12}. It is possible that these authors may have used a shorter incubation time than the 5-day period we have found necessary.

The conversion of glycine to methyl was confirmed by the use of [α-^{14}C]glycine and identification of labelled methionine as the major metabolic product of the ^{14}C-glycine in the cell protein hydrolysate. Both growth experiments and Warburg respiration experiments with resting cells indicated that glycine is poorly used for growth or for respiration. Comparison of radioautographs with the ninhydrin-sprayed chromatograms indicated that most of the amino acids synthesized arise from the glucose and ammonium chloride of the medium and not from the glycine. Formate was toxic for the organism at levels less than those used for glycine. Homocysteine plus choline were unable to replace methionine whether or not vitamin B_{12} was present, indicating the direct transformation of homocysteine to methionine after one-carbon transfer (e.g. via *S*-hydroxymethylhomocysteine) rather than methyl synthesis elsewhere (e.g. in betaine or choline) followed by transmethylation.

* John Simon Guggenheim Memorial Foundation Fellow, on leave of absence from the Division of Animal Nutrition, University of Illinois.

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Synthesis of Glutathione by Normal and X-irradiated Lens. By K. W. DAISLEY. (*Nuffield Laboratory of Ophthalmology, University of Oxford*)

The lens contains as high a concentration of reduced glutathione (GSH) as that recorded in any other tissue, and a progressive drop in GSH content can be correlated with loss of lens transparency; this was studied by Pirie, van Heyningen & Boag (1953) who used X-rays to initiate the opacity ('cataract') and GSH loss. The present work was undertaken to see if synthesis of GSH by the lens can be demonstrated, and if radiation causes a loss of this synthetic ability. The observation that the level of GSH begins to fall before any clinical signs of damage appear suggests that destruction of systems which maintain the level of GSH may prove to be an early result of irradiation.

Incorporation of [^{14}C]glycine into the GSH of intact lens in tissue culture was investigated by Kinsey & Merriam (1950). Johnston & Bloch (1949, 1951) and Samuels (1953) studied synthesis of GSH in cell-free aqueous extracts of liver and *Escherichia coli* respectively; a method of this kind was employed in the present work with lens.

It was found that dialysed extracts of normal cattle and rabbit lens synthesize GSH from glutamate, cysteine and glycine when incubated at 37° in the presence of adenosine triphosphate, Mg^{2+}, K^+ and a system to reconvert adenosine diphosphate to the triphosphate. Samples were taken at intervals for measurement of GSH content by the glyoxalase method of Woodward (1935). The GSH formed during incubation of rabbit lens extract represented 70–140 mg./100 g. lens wet weight/hr. at pH 6·4.

After X-irradiation of rabbit eye *in vivo*, this ability to synthesize GSH was lost. Decrease in synthetic activity was progressive over several months and appeared to run approximately parallel with the drop in GSH content during the course of cataract formation. The completely opaque lens neither contained GSH nor had the capacity to synthesize it.

It can be concluded that destruction of the glutathione-synthesizing enzymes of the lens is not a primary effect of irradiation, but progressive loss of this synthetic activity does result and may well be closely associated with the course of lens opacification.

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On the Mechanism of Tissue Swelling *in vitro*. By A. LEAF (introduced by H. A. KREBS). (*Department of Biochemistry, University of Oxford*)

It is known that tissues whose metabolism is interfered with will swell in isotonic saline media. If the solute concentration of the medium is sufficiently increased tissue swelling can be prevented. These findings have been interpreted as evidence that the intracellular fluids are normally hypertonic with respect to their extracellular fluid environment and that this hypertonicity is maintained by active extrusion of water from cells *in vivo* and from actively metabolizing cells *in vitro* (Robinson, 1950, 1953; Opie, 1949, 1950; Bartley, Davies & Krebs, 1954).

The validity of such an interpretation is entirely dependent upon the assumption that net movements only of water occur across the cell membrane during such *in vitro* experiments.

Experiments on guinea-pig kidney-cortex, rat cerebral-cortex and rat liver and rat liver mitochondria revealed that in all instances swelling was associated with movement of an approximately isotonic medium into the tissues. The quantity of sodium gained by the swollen tissue was accounted for by that which entered with extracellular anion plus that which exchanged on an ionic basis with intracellular potassium.

The dependence of fluid exchange of tissues on tissue metabolism can be explained on the requirement of metabolic energy for the maintenance of ionic gradients in tissues. Accumulation of sodium in cells when their metabolism is inhibited has as a consequence an increase of tissue chloride. The temporary rise in osmotic pressure within cells produced by such entry of ions of the medium manifests itself as an increase in tissue hydration. Re-establishing metabolic activity in the tissue will result in extrusion of sodium and chloride with reduction in tissue hydration. Because the effective osmotic pressure of polyvalent intracellular colloids is reduced by increasing the salt concentration of the medium, it is expected that

tissue swelling will be prevented in hypertonic saline media.

It seems unnecessary for the present to assume that the intracellular osmotic pressure within cells *in vivo* and in metabolizing cells *in vitro* is higher than that of the extracellular fluid in order to account satisfactorily for the observed fluid exchange of tissues *in vitro*.

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Some Biological Properties of the Urinary Mucoproteins. By A. J. Anderson, E. Lockey and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, University of London, London, S.W.* 1)

We have isolated three similar mucoprotein fractions (A_1, A_2 and A_3) from human male urine by adsorption on benzoic acid (Anderson & Maclagan, 1953, 1955*b*). Isolation of A_1 was carried out at pH 3 in 20% (v/v) acetone while isolation of A_2 and A_3 took place at pH 4·2 in aqueous medium. A_1 and A_2 were prepared at room temperature, and A_3 at 4°.

Yields of 2·6–3·9 mg./100 ml. were obtained with mobilities between serum α_1- and α_2-globulin (paper electrophoresis, pH 8·6). At pH 4·6, however, two negatively charged components were observed which may be similar to the electrophoretic serum fractions (M_1 and M_2) described by Mehl, Golden & Winzler (1949). The properties of these urinary mucoproteins, some previously reported (Anderson & Maclagan, 1953; Hewitt, 1953), indicate a wide range of biological activity.

All three fractions had amylase activity, 2 mg. of A_1, the most active, digesting 3 mg. of starch in 90 min. A_3 possessed 100–200 m.u.u./g. of gonadotrophic activity, A_2 being inactive. A_3 had some vitamin B_{12} binding capacity, 1 mg. binding 14 μg. of the vitamin (A. L. Latner, private communication). A trace of blood group substance activity was present in all three fractions (W. T. J. Morgan, private communication).

A_3 inhibited the influenza virus haemagglutination of red cells (H. B. Hewitt, private communication), its activity being comparable to the urinary mucoprotein fraction isolated by Tamm & Horsfall (1952). A_1 and A_2 were also inhibitory but were less active (Hewitt, 1953). Inhibition of blood coagulation by A_3 was noted, 8 mg. inhibiting completely the visible coagulation of 1 ml. of blood. Observations on the urine mucoprotein levels and the levels of Winzler's serum mucoprotein M_1 in various pathological conditions, using methods already described (Anderson & Maclagan, 1955*a*, *b*), revealed a negative correlation between these levels and certain flocculation tests for liver function (thymol turbidity, colloidal gold and zinc sulphate). This suggested that the mucoproteins act as inhibitors of these flocculation tests, and confirmation of this was obtained by addition of fraction A_3 to positive sera. Fraction A_3 (10 mg. %) also partially inhibited the precipitation of bovine serum albumin in urine (20 mg. %), both by heat and by sulphosalicylic acid.

It appears that in some of these reactions the mucoproteins act by inhibiting protein precipitation, probably functioning as protective colloids.

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A Medium for Liver Nuclei. By J. E. Stanier and J. St L. Philpot. (*M.R.C. Radiological Research Unit, Harwell*)

As a suspension medium for rat liver nuclei which keeps them normal for short periods as judged by phase-contrast and by solubility of nucleoprotein we recommend provisionally a solution containing 0·29M sucrose, 40% (v/v) glycerol, 0·005M potassium phosphate buffer of pH 6·8 and 0·001M $CaCl_2$.

Sucrose (Hogeboom, Schneider & Palade, 1948) prevents 'bleb' formation (Anderson, 1953*a*, *b*) possibly by preventing mitochondria from liberating harmful enzymes (Appelmans & de Duve, 1955). Glycerol prevents clumping of nuclei, possibly by lowering the dielectric constant and stabilizing DNA-histone. It may be replaced (for

short periods in the cold) by 20% acetone or 10% dioxan which have the same dielectric constant but which seem to denature DNA-histone slowly, making it insoluble in 1M-NaCl. Glycerol further permits storage at −80° indefinitely without apparent damage. For homogenization 40% glycerol may be replaced by 20% acetone, in which cells break more easily, followed by centrifugal layering into intermediate mixtures. Potassium is probably replaceable by sodium. Phosphate buffer is probably natural, though its ionic strength per mole (about 2 at pH 6·8) is undesirably high. Bicarbonate-CO_2 is undesirably volatile. Veronal causes DNA to swell out through the nuclear membrane. Lutidine seems to cause (or not to prevent) slow nuclear emptying without swelling. The pH 6·8 is natural, but 7·6 apparently gives cleaner preparations (Anderson, 1955) and less emptying. The ionic strength ($\sim$0·01) is sufficient to prevent swelling of the whole nucleus (including its membrane) and to allow adequate buffering, without causing excessive mitochondrial clumping. The concentration of calcium chloride is sufficient to prevent disappearance of the nucleolus without causing nuclear granularity, at the chosen ionic strength. At higher ionic strengths (which may be used when mitochondrial clumping does not matter) the calcium concentration must be lower to avoid nuclear granularity, and is best regulated by a calcium buffer based on nitrilotriacetic acid ($pK_{Ca} = 3{\cdot}48$ at pH 6·8). Calcium may be replaced by magnesium or barium.

Physicochemical comparison of the above medium with cell sap awaits isolation of the latter.

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DEMONSTRATION

A 'Spectral' Centrifuge for Fractionating Cell Particles. By J. St L. Philpot and D. A. Stock. (*M.R.C. Radiobiological Research Unit, Harwell*)

A suspension of cell particles is fed continuously into a drum centrifuge, together with a heavier liquid on which it floats. The particles sediment outwards on their way up the drum, producing a sedimentation spectrum on the wall, which has a barbed shape to support the sediment after stopping. Alternatively, fractions may be discharged through suitable holes at the top of the drum as in the Sharples centrifuge. The drum is supported on a spherical air bearing and is driven by a reaction turbine fed axially through the bearing.

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